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GREENHEART ALKALOIDS

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Type C   Final Report

by

Peter J. Hearst, Ph. D.

ABSTRACT

It is commonly believed that the resistance of the tropical wood greenheart (Ocotea rodiaei) to marine borer attack is due to the pressence of the toxic alkaloid "bebeerine," and that this alkaloid is the same as chondrodendrine (I), whose structure is known. The latter alkaloid could not be obtained from greenheart. Rodiasine dimethiodide, which was isolated, apparently is the first pure alkaloid obtained from greenheart. By gradient elution chromatography and subsequent fractional crystallization, eight alkaloid hydrochlorides were isolated from the ether-soluble alkaloids of greenheart bark. The alkaloids fall into two groups. One group consists of four bisbenzylisoquinoline alkaloids of the oxyacanthine series: ocoteamine (XV) and otocamine (XVII), which are, respectively, one of the methyl ethers and the dimethyl ether of trilobamine; and demerarine and ocodemerine, which appear to be the corresponding diasterioisomers. The other group consists of four alkaloids which have properties of bisbenzylisoquinoline alkaloids with one diphenyl ether linkage: rodiasine, isorodiasine, and norrodiasine, which all have the same skeletal structure; and dirosine, which has a different skeletal structure. All the alkaloids are quite toxic to Teredo but less toxic to Limnoria, and when impregnated into pine panels, they prevent Teredo attack and greatly reduce Limnoria attack. The alkaloids may therefore be the chief factor in the resistance of green-heart to the former species of marine borer but may be a less important factor in its resistance to the latter species.

FIGURES

FORMULAS

I. INTRODUCTION

Marine wood-boring organisms cause considerable damage to wooden structures in sea water and are responsible for considerable expenditures by the Naval Shore Establishment. This Laboratory has been conducting investigations to develop improved methods of treating timbers to prevent or reduce marine borer attack.

In order to combat marine borer attack effectively, a fundamental knowledge of the marine boring organisms, of their behavior, and of the materials which are toxic to them or repel them, is important. Greenheart is very resistant to marine borer attack, and a better knowledge of its toxic or repellent constituents was therefore considered to be of importance. If the constituents were highly effective, a knowledge of their composition might lead to the development of new protective agents. Furthermore, this knowledge might lead to the development of tests which could reliably predict the resistance and the expected life span of new piling.

Initially, the problem was to isolate from greenheart or to otherwise obtain the presumably known alkaloid "bebeerine," to test its toxicity to marine boring organisms, and to synthesize related compounds which might be potential protective agents for wooden piling. Later, it became obvious that the ether-soluble alkaloids were a complex mixture whose constituents were not known, and the immediate problem was to determine the structures of the alkaloids of greenheart.

This report presents the results of an investigation of the toxic alkaloids of greenheart.

II. HISTORICAL BACKGROUND

A. Resistance of Greenheart to Marine Borer Attack

Damage by marine wood-boring organisms can be reduced by impregnating with toxic agents those timbers which otherwise would be readily attacked or by employing timbers which are naturally resistant to marine borer attack. In the United States, wooden piers are most often constructed of Douglas fir or of southern yellow pine, impregnated with creosote. In northern Europe and in some areas of the tropics the use of naturally resistant woods is more common.

Naturally resistant timbers have been used since the beginning of history. Noah's ark supposedly was built of "gopher wood."[1] The latter wood was the cypress which grew in large forests in Biblical days and because of its durability was used for shipbuilding. Another cypress, the Australian cypress pine, was one of the 20 most resistant of more than 300 species tested by Edmondson[2] for marine borer resistance. The resistance of the latter species was believed to be due to the presence of callitric acid, which is toxic to fungi and repellent to termites.[3] However, there may not be a close relationship between the Australian cypress pine, Callitris glauca, and the "gopher wood," which is believed to have been a species of Cypressus.[1]

Presently, the most widely used of the naturally resistant timbers is probably greenheart. Greenheart was first imported to England from British Guiana in about 1770. Like most other naturally resistant woods, it is a tropical wood. It is grown commercially in British Guiana and is often called Demerara greenheart. The principal native names of the wood are given as bibiru and sipiri. The systematic name is Ocotea rodiaei, sometimes spelled Ocotea rodioei, but the name Nectandra rodioei is sometimes used. There is little question concerning the correctness of the first-named designation.[4]

The high resistance of greenheart to marine borers has widely been attributed to the presence in greenheart of the alkaloid "bebeerine."[4-7] Other factors which have been claimed to contribute to this resistance are the presence of resinous tyloses, or ingrowths, and the texture and hardness of the wood.[6] The silica content of greenheart is very low and therefore is not believed to contribute to its resistance.

The protective action of "bebeerine" finds its chief scientific support in the work of Barger, reported in 1924.[7] Blocks of Baltic fir were impregnated with an alcoholic extract of greenheart sawdust and were found to resist Teredo attack for two seasons. The alcoholic extract was reported to contain 1/10 of 1% of a non-crystalline alkaloid corresponding to "bebeerine." Barger concludes: "It seems therefore that greenheart owes its protection not to its mechanical nature, but to a chemical constituent soluble in alcohol (probably the alkaloid bebeerine)."

B. Chemistry of Greenheart Alkaloids

In 1843, Maclagan[8] reported the presence of an ether-soluble alkaloid and of an ether-insoluble alkaloid in greenheart bark. These he called bebeerine and sipeerine, respectively, after the Indian and Dutch names of the tree. In 1851, von Planta[9] reported for bebeerine a formula equivalent to $C_{18}H_{21}O_3N$. In 1869, Maclagan and Gamgee[10] reported the presence of a chloroform-soluble alkaloid, nectandria, of a chloroform-insoluble alkaloid, and of a third base, in greenheart wood. In the same year Flückiger[11] claimed that bebeerine was identical with an alkaloid obtained from Pareira brava.

Since 1869, all the reported chemical investigations of "bebeerine" have been performed with material obtained from Pareira brava or related plants. No further investigations of alkaloids definitely from greenheart have been reported between that time and the beginning of the present investigations, except for the brief work of Barger.[7]

Authoritative reviews of alkaloid chemistry[12, 13] claim that "bebeerine" from greenheart and from Pareira brava and Chondrodendron* species are identical, even though the validity of Flückiger's comparison was challenged by Faltis and Neumann.[14] The latter believed that the alkaloid from Pareira brava is not the same as that from greenheart, and suggested that it be called chondrodendrine.* However, the term "bebeerine" has continued to be employed for the alkaloid from Pareira brava and from Chondrodendron species by recent workers in this field. Although this alkaloid

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* Although earlier usage generally employs the term Chondodendron, it has been claimed that Chondrodendron is more correct.[55] Both terms appear to be considered proper usage, and similarly, both chondodendrine[12, 14, 15, 76] and chondrodendrine[13, 33, 52, 55, 71, 77] appear in the literature.

is generally dextrorotatory, it has also been isolated in the racemic and in the levorotatory forms. The latter has been found to be identical with the alkaloid curine, [15] which has been isolated from tubo-curare, [16] one of the arrow poisons prepared by the South American Indian tribes of the upper Amazon from extracts of local plants. The structure of "bebeerine" was proposed by Späth[17] in 1934 and was confirmed by King[18] in 1939. This alkaloid has the structure represented by formula I.
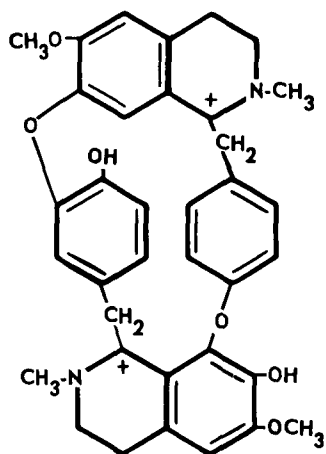
I

III. EXPERIMENTAL WORK*

A. Alkaloids From Pareira Brava

In an effort to obtain chondrodendrine, or "bebeerine," two shipments of Pareira brava root were extracted with tartaric acid and the free bases from the extracts were extracted with ether according to procedures employed by others.[20-22] Chondrodendrine, which is reported to crystallize from a methanolic solution of the ether-soluble alkaloids, could not be isolated.

Commercial "Bebeerine Hydrochloride," which could not be obtained in this country, was obtained from Scotland. However, this material was not the hydrochloride of the desired compound, but was instead the hydrochlorides of the total alkaloids from Pareira brava. Again, no "bebeerine" could be isolated. It was possible to isolate the known compound isochondrodendrine by crystallization, from a chloroform - methanol solution, of the chloroform extract of the free bases. (See further Section III.L.1.)

B. Properties of the Greenheart Material

The greenheart material was supplied directly from British Guiana, in three shipments designated (W), (G), and (G2). Included in these shipments were bark, sawdust, roots, seeds, and leaves.

The bark was obtained in mixed pieces about 1/4 inch thick and averaging 2 x 5 inches in size. The sawdust was obtained directly from the mill.

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* Temperatures are in degrees Celsius (centigrade);[19] melting points are corrected; specific rotations are given for temperatures of 22-26° and for sodium light, unless otherwise specified. (The abbreviation, $[\alpha]_D$, indicates the specific rotation determined with sodium light; and the notation, $[\alpha]_{5461}^{22}$ +283° ($\underline{c}$ 1.023 in water), indicates a specific rotation of +283° determined with the green mercury line at a temperature of 22° and a concentration of 1.023 g. per 100 ml. of water.)

The following analyses were obtained for dried bark and sawdust:

Material	Nitrogen, %	Ash, %
Bark (W)	0.83	15.5
Bark (G)	0.65	8.6
Sawdust (W)	0.44	0.47
Sawdust (G)	0.56	0.46

Spectrographic analyses of the ashes of bark (W) and of sawdust (W) showed the presence of a variety of trace elements, including copper. The calculated copper contents of the original bark and sawdust were 1 and 1.6 parts per million, respectively.

C. Extraction of the Alkaloids

The methods employed for the extraction of the alkaloids were modified from the procedure used by King[20] for the alkaloids of Pareira brava. The bark or sawdust was first ground in a Wiley Mill to pass a 2-mm. screen. Initial extractions of 3- to 4-kg. quantities of bark or sawdust were carried out in a 10 x 100-cm. glass tube by percolating the ground material with approximately 20 liters of 1% tartaric acid. The filtered extracts were evaporated in vacuo to approximately 2.5 liters and were alkalized to pH 9 to precipitate the crude bases, which were collected and dried in vacuo over calcium chloride.

Extractions of 10-kg. quantities of bark or sawdust were performed by an agitation and percolation procedure. To the material was added 17.5 liters of 1% aqueous d-tartaric acid. The mixture was stirred rapidly for 2-1/2 hours and was allowed to stand overnight. The supernatant liquid was filtered, was concentrated to approximately one-fifth its original volume, and was refiltered. Sodium hydroxide was added to the cooled solution to give pH 5. Saturated aqueous sodium carbonate was added until pH 9 was reached. The precipitate was collected, washed with water, and dried under reduced pressure over sodium hydroxide. The extraction of the bark was repeated with nine additional 7.5-liter portions of aqueous tartaric acid. The final extract was obtained by suction filtration. The total yield of crude alkaloids was 220-240 g.

Large-scale extractions of 50-kg. quantities of bark were carried out by modifying the above procedure. Six extractions were made with 80-liter portions of 0.1 N sulphuric acid. The extracts were not concentrated but were alkalized directly to pH 9 with sodium hydroxide and sodium carbonate. The precipitated crude bases were collected and dried.

The yields of crude bases in the moderate-scale extraction was 2.3% for bark (G) and 1.5% for bark (W). Sawdust (G) gave 3.2% and sawdust (W) gave 2.8% of crude bases. Large-scale extractions of bark (G2) gave a 2.0% yield of crude bases.

The crude bases were exhaustively extracted with ether in a Soxhlet apparatus to give the "ether-soluble" alkaloids. The extract was collected after two to four days of extraction. It was dissolved in dilute hydrochloric acid, washed with ether, reprecipitated with ammonia, and collected and dried to give the ether-soluble alkaloids. (In later large-scale extractions the alkaloids were not precipitated with ammonia but were freeze-dried to give the dry alkaloid hydrochlorides.) The ether extractions were continued for eight or more days to give smaller quantities of less ether-soluble alkaloids, which were worked up in the same manner.

The yields of ether-soluble alkaloids from the crude bases of the bark generally were 25 to 40%. The crude bases from the sawdust gave yields of 8 to 17%.

The over-all yield of ether-soluble alkaloids from the bark was thus approximately 0.5 to 0.8%. The yield from the sawdust was approximately 0.4%.

D. Properties of the Bark Alkaloids

The mixture of ether-soluble alkaloids was an amorphous, almost white powder melting in the region of 180-215°. The mixture had a specific rotation of approximately +129° in methanol.

The ether-soluble alkaloids gave positive tests for nitrogen and negative tests for sulphur, halogen, or phosphorus. Negative tests were obtained for aldehydes (with Schiff reagent and dimedone),[23] for esters (with the ferric hydroxamate test),[24] for methylenedioxy groups (with the Labat test),[25] for enols (with ferric chloride),[26] and for phenols with free para positions (with the indophenol test).[27] Positive tests for phenols were obtained with Folin reagent[28] and Millon's reagent.[29]

Anal. Calcd. for $C_{18}H_{21}O_3N$: C, 72.21; H, 7.07; N, 4.68; $2CH_3O$, 20.7. Calcd. for $C_{36}H_{38}O_6N_2$: C, 72.70; H, 6.44; N, 4.71; $2CH_3O$, 10.4. Found for ether-soluble alkaloids (G): C, 72.79; H, 6.23; N, 4.45; CH_3O, 19.5.

The ether-soluble alkaloids were very soluble in methanol. They appeared to be a complex mixture. Many fractional crystallizations from a variety of organic solvents and solvent mixtures gave various fractions of different properties, with melting points from 120° to 255°, but no crystalline material could be isolated.

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One percent solutions of the alkaloids in 6 $\underline{N}$ hydrochloric acid or in 20% methanolic potassium hydroxide did not change in rotation on heating to 50° for 26 hours or on refluxing for 22 hours, respectively. However, a sample in methanol stored in a refrigerator for several months showed a decrease in specific rotation from +125° to +73°, and samples in benzene gave similar decreases. A sample in methanol kept in a polarimeter tube for a month gave an initial decrease from +120° to +110° without any further change. Reexposure to air gave a further decrease to +89° in a period of 2-1/2 weeks.

Extraction of the alkaloids with benzene and subsequent crystallization attempts gave fractions of decreased specific rotations and of decreased solubilities. Samples kept in chloroform or methylene chloride overnight became partly insoluble and gained in total weight.

The equivalent weight of the ether-soluble alkaloids from bark (G) was approximately 316 as determined by three methods: (a) A 200-mg. sample was dissolved in water containing an excess of hydrochloric acid and the solution was titrated with 0.100 $\underline{N}$ sodium hydroxide. The volume vs. pH curve had two inflections differing by 6.28 ml. The calculated equivalent weight was therefore 318. (b) A 3.511-g. sample in methanol was treated with excess methyl iodide and after the reaction was completed the solvents were removed to give 5.092 g. of quaternary alkaloids. The uptake of methyl iodide corresponded to that of a tertiary amine with an equivalent weight of 315. (c) On the basis of the above nitrogen determination (4.46%), the calculated equivalent weight is 314.

The ether-soluble alkaloids prevented the normal precipitation of silver and copper as the halide and sulphide, respectively. Isochondrodendrine had a similar effect. Ten milligrams of the greenheart alkaloids were dissolved in 2 ml. of water containing two drops of 5% hydrochloric acid. Ten milligrams of isochondrodendrine were similarly dissolved. One drop of 2% silver nitrate solution gave no precipitate when added to either of these solutions but gave an immediate precipitate when added to a blank solution containing only the hydrochloric acid. To a second set of solutions there was added, instead of the silver nitrate solution, one drop of 5% sodium sulphide solution and one drop of 5% copper sulphate solution. The alkaloid solutions gave no precipitates but gave only light-brown, slightly colloidal, solutions, whereas the blank gave an immediate dark-brown precipitate.

E. Properties of the Wood Alkaloids

The ether-soluble alkaloids from sawdust (W) had a specific rotation of +16° in one-tenth normal hydrochloric acid. In the same solvent the ether-soluble bark alkaloids had a specific rotation of +120°. The equivalent weight of the ether-soluble wood alkaloids was approximately 314 based on the hydrochloric acid required to dissolve the amorphous alkaloids and on the increase in weight on reaction of the alkaloids with methyl iodide.

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F. Quaternary Derivatives

1. Rodiasine Methosalts

1a. Rodiasine Dimethiodide. — Treatment of 3.511 g. of ether-soluble alkaloids from bark (G) in 250 ml. of methanol with 14 ml. of methyl iodide at room temperatures, and partial evaporation of the solvents, gave 0.261 g. of colorless cubic crystals. The yield was 5.1% of a total yield of 5.092 g. of quaternary methiodides. Recrystallization from methanol gave a product decomposing at 321° in an evacuated capillary and having $[\alpha]_D$ +68° and $[\alpha]_{5461}$ +83° (c 0.147 in water). The ultraviolet absorption spectrum of an aqueous solution gave a peak at 283 mμ, a minimum at 259 mμ, and a gradual rise to 220 mμ without a peak at 225 mμ. This material was called rodiasine dimethiodide.

Anal. Calcd. for $C_{40}H_{50}O_6N_2I_2$: C, 52.87; H, 5.55; N, 3.08; I, 27.94; $4CH_3O$, 13.66; $4N$-CH_3, 6.62. Found: C, 52.95; H, 5.17; N, 3.26; I, 28.08; CH_3O, 13.79, N-CH_3, 6.75. The alkyl iodide from the alkoxyl determination was entirely methyl iodide, as determined by infrared spectra by the Clark Microanalytical Laboratories, Urbana, Illinois.

1b. O-Methylrodiasine Dimethiodide. — A 182-mg. portion of the above product in 25 ml. of methanol was treated with 0.8 ml. of 0.5 N methanolic potassium hydroxide and 0.08 ml. of methyl iodide under reflux for one hour, and this treatment was repeated seven times. Evaporation to dryness and recrystallization from water gave a product which decomposed at 304° in an evacuated capillary and had $[\alpha]_D$ +50° and $[\alpha]_{5461}$ +63° (c 0.255 in water). The ultraviolet absorption spectrum of the product, shown in Figure 1, did not differ appreciably from that of the starting material.

Anal. Calcd. for $C_{41}H_{52}O_6N_2I_2$: C, 53.37; H, 5.68; $5CH_3O$, 16.82. Found: C, 53.39; H, 5.39; CH_3O, 16.50.

1c. Rodiasine Dimethochloride. — An aqueous solution of the dimethiodide was treated with an excess of silver chloride, and the solution was filtered and evaporated. The product was recrystallized from isopropyl alcohol-water to give needles, $[\alpha]_D$ +81.5° and $[\alpha]_{5461}$ +100° (c 0.98 in water), melting at 286° with decomposition. This material gave a negative Millon's test.

Anal. Calcd. for $C_{40}H_{50}O_6N_2Cl_2$: C, 66.20; H, 6.94. Found: C, 66.06; H, 6.70.
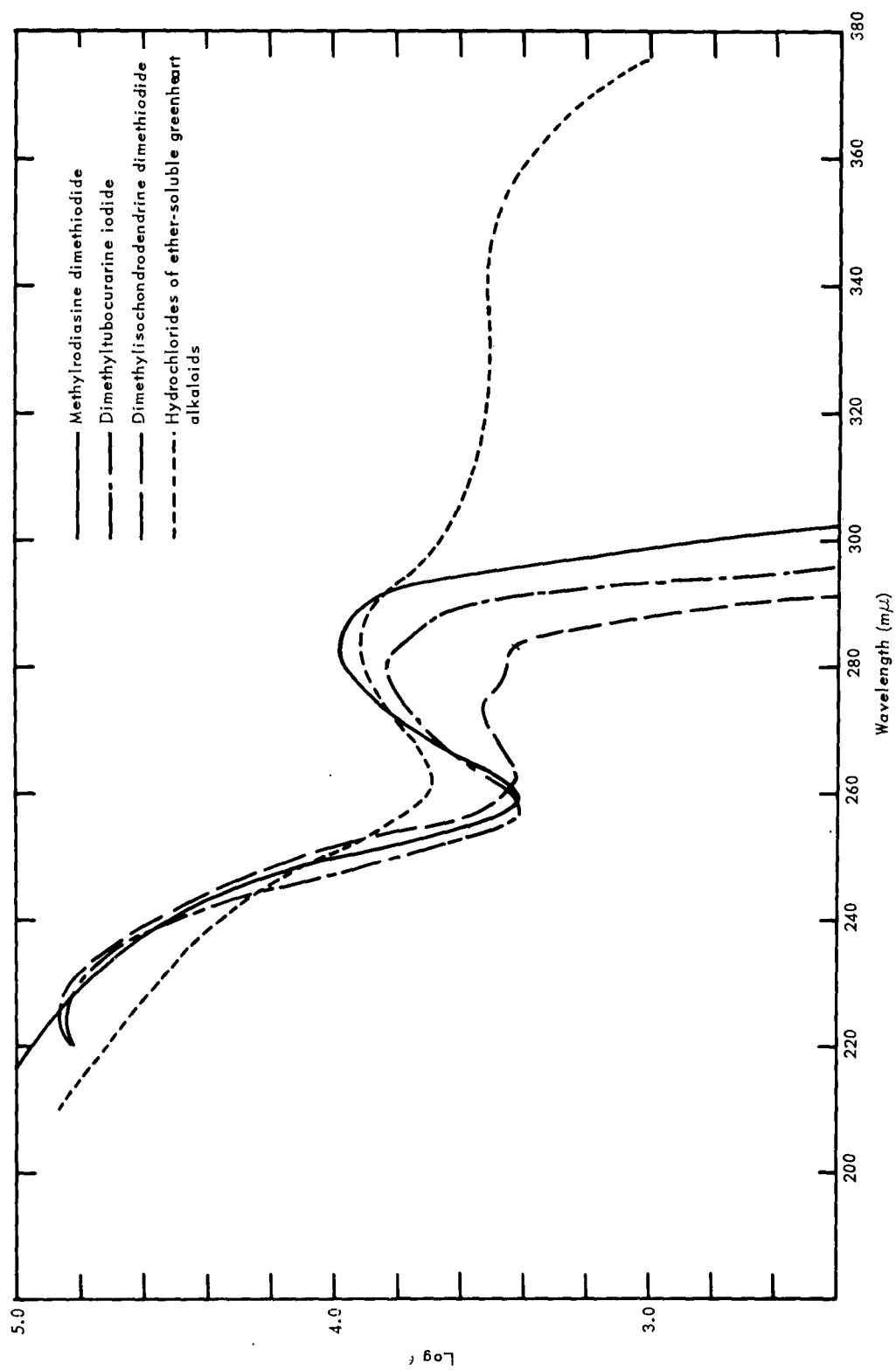
Figure 1. Ultraviolet spectra of some alkaloids (in 10^{-5} $\underline{M}$ and 10^{-4} $\underline{M}$ aqueous solutions).

Methylrodiasine dimethiodide
Dimethyltubocurarine iodide
Dimethylisochondrodendrine dimethiodide
Hydrochlorides of ether-soluble greenheart alkaloids

Wavelength ($m\mu$)

Log ϵ

10

1d. Rodiasine Dimethopicrate. — The picrate was prepared from the dimethiodide by treatment of an aqueous solution with a saturated aqueous solution of picric acid. The product, recrystallized from aqueous methanol, was obtained in two forms, melting at 180° and at 252°. The high-melting form could be converted to the low-melting form by crystallization and seeding with the low-melting crystals, and the low-melting form could similarly be converted to the high-melting form.

2. Rodiasine (W) Methosalts

2a. Rodiasine (W) Methiodide. — Treatment of the ether-soluble bark alkaloids from bark (W) with methyl iodide gave a methanol-insoluble product, rodiasine (W) methiodide, decomposing at 315°. There was no depression in melting point on mixing with rodiasine dimethiodide. The specific rotation, the ultraviolet absorption, and the X-ray diffraction pattern of this lower-melting methiodide were experimentally indistinguishable from the corresponding properties of rodiasine dimethiodide. Methylation of rodiasine (W) methiodide with methyl iodide and methanolic potassium hydroxide gave the O-methyl derivative, melting at 304° with decomposition. The latter had a specific rotation, an ultraviolet absorption spectrum, and an X-ray diffraction pattern indistinguishable from those of O-methylrodiasine dimethiodide, and it also had the same decomposition point.

2b. Rodiasine (W) Methochloride. — Rodiasine (W) methiodide (290 mg.) was converted to the methochloride by treatment with silver chloride. Evaporation to 4 ml. gave 12 mg. of a solid, melting at 309° with decomposition, and complete evaporation under reduced pressure gave 214 mg. of a partly crystalline mass decomposing at 299°. Crystallization of the latter from isopropyl alcohol - water gave 171 mg. of crystals decomposing at 294°, and 21 mg. of fine needles decomposing at 283°. The fine needles were dissolved in 1 ml. of water, and a few drops of 20% potassium iodide were added to precipitate the methiodide. The latter was recrystallized from methanol to give 18 mg. of rodiasine dimethiodide, melting at 320° with decomposition.

3. Other Methiodides

No other crystalline methiodides could be obtained from the methanol-soluble methiodides by crystallizing from water. The products obtained all had decomposition points of about 262° and specific rotations ranging from +13° to +33°. O-Methylation with methyl iodide and methanolic sodium methoxide gave a product which also could not be crystallized from water. The five major fractions obtained decomposed at temperatures ranging from 249° to 256°.

G. Direct Crystallization of Hydrochlorides

1. Alkaloid B Hydrochloride

A number of batches of the ether-soluble alkaloids had been stored in dilute hydrochloric acid in a refrigerator for prolonged periods. In some instances needles slowly deposited over periods of one to six years. For example, from one solution containing approximately 11 g. of alkaloid hydrochlorides (W), which had remained in the refrigerator for more than a year, there were obtained 426 mg. (4%) of crystals; $[\alpha]_D$ +225°. Recrystallization to constant rotation gave needles of alkaloid B hydrochloride; $[\alpha]_D$ +248° and $[\alpha]_{5461}$ +301° (c 0.99 in water); m.p. 287° dec.

The fractional crystallization of 15 g. of alkaloid hydrochlorides (G) was attempted by adding progressive amounts of hydrochloric acid to an aqueous solution, and collecting the solid or oily precipitates. The precipitates were again dissolved and reprecipitated in a complicated scheme involving about 25 precipitations. Most of these contained only a few crystals or none, but a 284-mg. portion and a 400-mg. portion of predominantly crystalline material (4.6%) were obtained. Both of these on recrystallization gave alkaloid B hydrochloride; $[\alpha]_D$ +251° and $[\alpha]_{5461}$ +300° (c 1.00 in water); m.p. 285° dec.

In some instances it was possible to obtain a crystalline residue when batches of the ether-soluble alkaloids were dissolved in 6 N hydrochloric acid. Such residues on recrystallization gave alkaloid B hydrochloride.

2. Alkaloid C Hydrochloride

One solution of ether-soluble alkaloids (G) in dilute hydrochloric acid gave 1.3 g. of a precipitate which was not completely soluble in 30 ml. of water. The suspension of needles was separated from lint by dissolving in methanol. Evaporation to dryness gave 0.46 g. of amphoteric material very slightly soluble in either dilute hydrochloric acid or in sodium hydroxide solution. Recrystallization from very dilute hydrochloric acid containing a little acetone and again from very dilute hydrochloric acid gave alkaloid C hydrochloride; $[\alpha]_D$ +72° and $[\alpha]_{5461}$ +90° (c 1.00 in water); m.p. 283° dec.

H. Countercurrent Distribution

A countercurrent distribution of the ether-soluble alkaloids was run with a 54-tube stainless-steel Craig countercurrent distribution apparatus.[30] A system consisting of 0.5 M acetate buffer, pH 4.20, and chloroform was employed. Each tube held approximately 9 ml. of each phase, and the sample at a concentration of

1 mg. per ml. was introduced in the upper layer of tube No. 0. The transfers were made in the usual manner. At the end of the run, all upper layers were treated with ammonia and, by shaking the apparatus, the alkaloids were transferred to the lower layer. The absorbances of the lower layers were determined and from these values the curve was plotted. Three major peaks and one minor peak were obtained as shown in Figure 2. The distribution coefficients, calculated from the positions of the maxima of these peaks, [31] were 0.09, 2.5, 10, and 0.45, respectively.
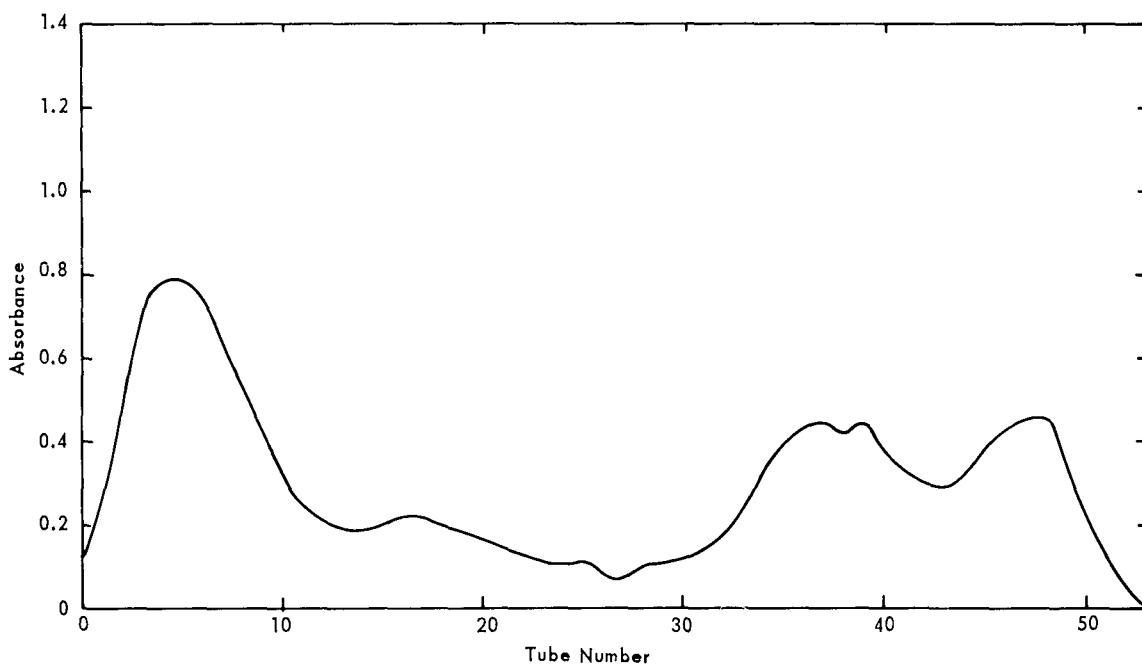
Figure 2. Countercurrent distribution of greenheart alkaloids.

With this same solvent system a preparative scale countercurrent distribution
was run. Eleven 1-liter separatory funnels and 450-ml. solvent phases were employed.
Ten grams of the hydrochlorides of the ether-soluble alkaloids were subjected to ten
transfers. At the end of the run the aqueous layers were made alkaline and the pro-
ducts were extracted into the chloroform layers. The absorbances of the chloroform
layers, diluted 1:25, were determined and were plotted as percent of the total
absorbance, as shown in Figure 3. The solvents were evaporated from fractions 2
through 9, 25-ml. portions of 1% hydrochloric acid were added to each fraction,
and the evaporations were completed. Additional hydrochloric acid was added to
the hydrochloric acid solutions and these were set aside to crystallize. The crystals
formed were collected by filtration and were dried and weighed. The amounts
obtained, plotted as percentages of the 10 g. of starting material, are shown in
Figure 3.
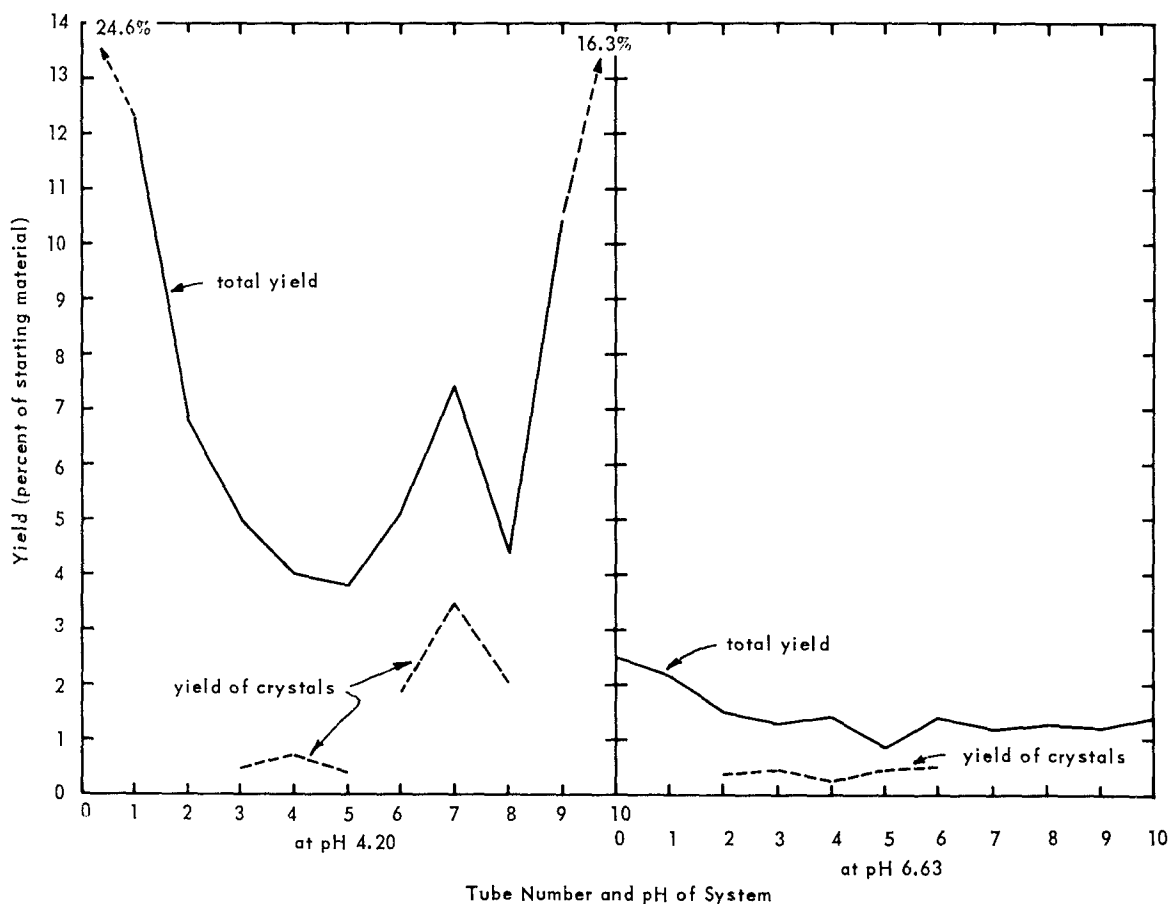
Figure 3. Preparative scale countercurrent distribution.

The 1.5 g. of alkaloids in tube No. 10 were subjected to another ten-transfer distribution. Two-hundred-milliliter phases of a phosphate buffer, pH 6.63 - benzene system were employed. At the end of the run the alkaloids were brought into the benzene layers by alkalization and the absorbances of the 250-ml. benzene solutions, diluted 1:20, were determined. From the absorbances of the fractions, the approximate percentages of the 10 g. of starting material were determined, and these percentages were plotted as shown in Figure 3. The benzene solutions were evaporated to small volumes, 20-ml. portions of 0.5% hydrochloric acid were added, and the evaporation of the benzene layers was completed. On prolonged standing, crystalline needles were obtained from fractions 2 to 5 and irregular flat plates from fractions 5 and 6. The weights of these small amounts of crystals are plotted in Figure 3.

The crystals from fractions 3, 4, and 5 of the first distribution totaled 155 mg. They were combined and were recrystallized from 15 ml. of 0.5% hydrochloric acid to give 99 mg. of alkaloid D hydrochloride; $[\alpha]_D$ +73° ($\underline{c}$ 1.00 in water). The fact that this alkaloid was different from alkaloid C hydrochloride of the same specific rotation was shown by the respective distribution coefficients of 0.70 and 2.0, determined for 0.5 $\underline{M}$ acetate buffer, pH 4.17 - chloroform as described below in Section III. J. 2.

The 749 mg. of crystals from fractions 6, 7, and 8 were combined and were recrystallized three times from 30-ml. portions of 2% hydrochloric acid to give 564 mg. of alkaloid E hydrochloride; $[\alpha]_D$ +97°, $[\alpha]_{5461}$ +114° ($\underline{c}$ 1.02 in water).

I. Chromatographic Separation

1. Conventional Column Chromatography

Neutral alumina was prepared from Alcoa F-20 alumina by treating with sulphuric acid at pH 2 to 3 for one hour, washing thoroughly with water by decantation, treating with ammonia at pH 9 for one-half hour, washing with water, washing with alcohol, drying under reduced pressure, and finally heating at 210° at atmospheric pressure for two hours. The alumina was deactivated by exposure to the atmosphere, and the activities were determined by the method of Brockmann.[32] An aqueous suspension of this alumina had a pH of approximately 7.0.

A preparative scale chromatography was run as follows: Ten grams of ether-soluble alkaloids (G2) were chromatographed on a 5.2 x 15-cm. column of approximately 300 g. of neutral alumina of an activity midway between III and IV. The 19.1 liters of eluent employed consisted of 5.0 liters of methylene chloride, 3.4 liters of 0.5%, 2.2 liters of 1%, 3.5 liters of 2%, 2.0 liters of 5%,

and 2.0 liters of 20% methanol in methylene chloride, and finally 1.0 liter of methanol. The chromatography was followed by taking 1-ml. samples of the eluent stream at 250-ml. intervals, diluting the samples 1:10 with 1% methanol in methylene chloride, and determining the absorbances of these solutions in 1-cm. cells at 283 mμ. The values obtained were multiplied by 10 and plotted to give the curve shown in Figure 4.

The eluate was divided into 12 fractions in what appeared according to the curve to be the most logical manner. The fractions were evaporated to approximately 50 ml., 50-ml. portions of water were added, and the mixtures were acidified with small excesses of hydrochloric acid. The fractions were further heated on a steam bath and flushed with a stream of nitrogen to remove the remaining methylene chloride, and the resulting solutions were freeze-dried. The total recovery was 94.2%. Fractional crystallization from dilute hydrochloric acid of the fractions eluted with methylene chloride and with methylene chloride containing 0.5% methanol, gave alkaloid D hydrochloride and alkaloid E hydrochloride, respectively. Other fractions eluted with up to 5% methanol gave other crystalline products which were not completely purified.
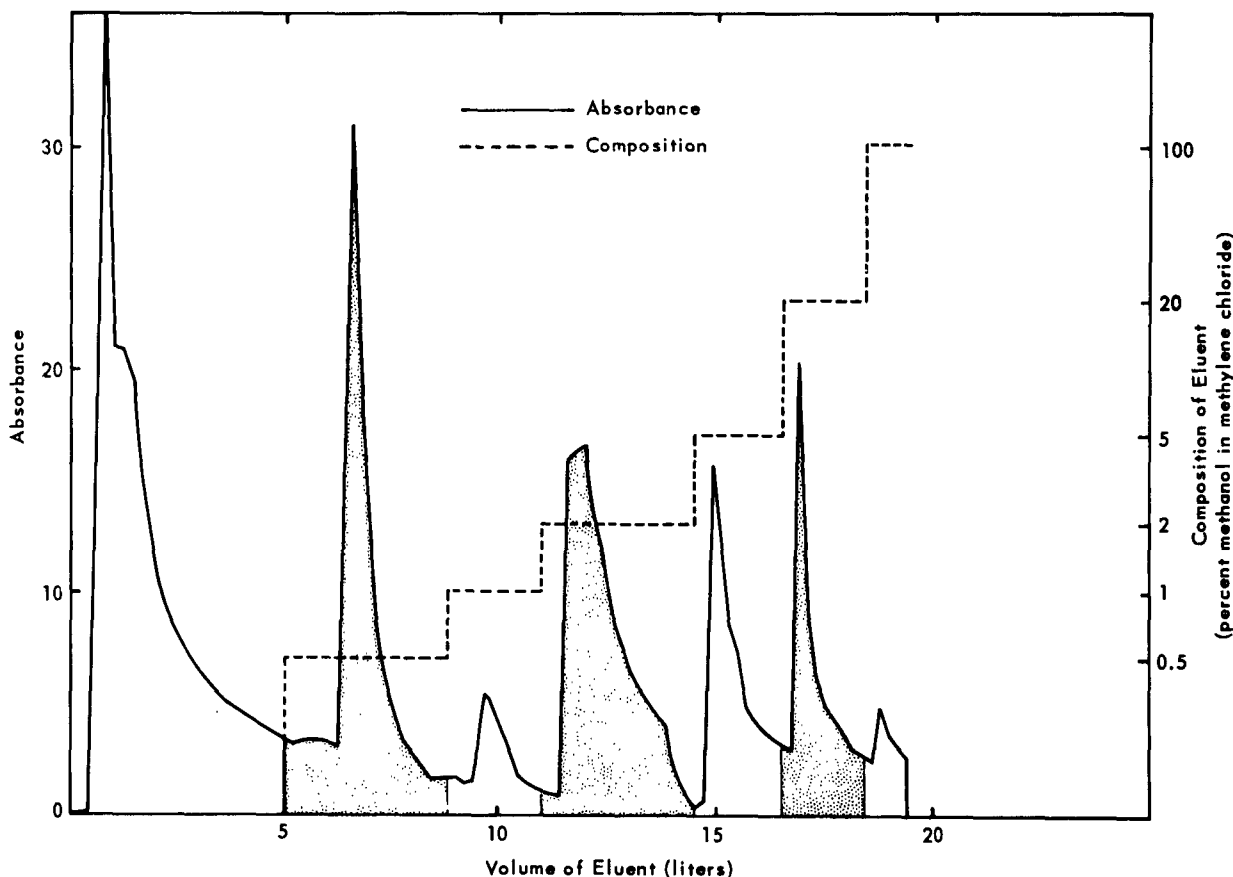
Figure 4. Chromatography of greenheart alkaloids.

2. Gradient Elution Chromatography

Thirty grams of ether-soluble alkaloids (G2) were chromatographed on a 7.5 x 18-cm. column of approximately 750 g. of neutral alumina of activity III. The eluent employed consisted of 2.75 liters of methylene chloride (including the methylene chloride in which the alkaloids had been dissolved), followed by the 22 liters of gradient eluent. The latter had the composition shown in Figure 5 and was prepared as described in Appendix A. The chromatography was completed in about eight hours. At 250-ml. intervals, eluent samples were taken and diluted 1:100, and the absorbances of these solutions were determined at 283 mμ. For a typical run, the observed absorbances multiplied by 100 are plotted in Figure 5.
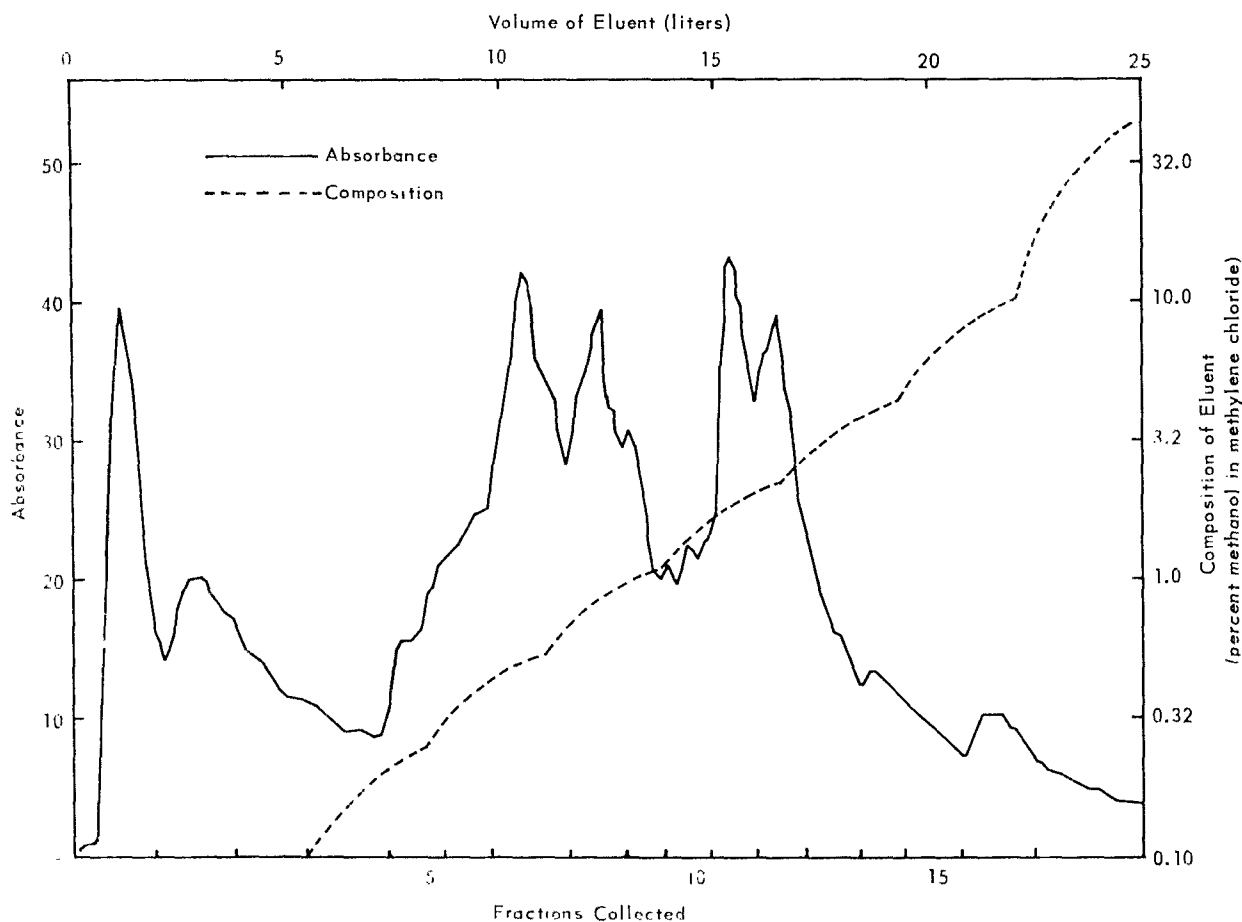
Figure 5. Gradient elution chromatography of greenheart alkaloids.

Three runs carried out under similar conditions gave curves which were quite similar to each other. The fractions from these runs were cut in similar manners, employing as guides the shapes of the curves as well as the cumulative absorbances and the cumulative volumes of the eluates. The fractions were converted to the alkaloid hydrochlorides as described above, and the 17 corresponding fractions from each of the three runs were combined. Since the equivalent weight of the 90 g. of starting material was about 315, the theoretical yield was 100 g., and the over-all yield of 91 g. was a 91% recovery. The properties of the fractions are listed in Table I.

In a subsequent set of chromatographies, 200 g. of ether-soluble alkaloids (G2) were chromatographed in four 50-g. portions. Woehlm alumina, nonalkaline, was employed in 1800-g. portions, deactivated with 40.5 ml. of water to give an activity somewhat greater than II. The columns were approximately 9.4 x 28.5 cm. The eluent employed in each chromatography consisted of 3 liters of methylene chloride, followed by 38 liters of gradient eluent. The latter was prepared by adding to the 5.5-liter reservoir filled with methylene chloride, 2-liter portions of 0.25% methanol in methylene chloride, and succeedingly more concentrated solutions of methanol in methylene chloride. The concentrations of these solutions varied by a factor of the fifth root of four. The eluate from the column was run through a flow cell having a 0.15-mm. path length. The latter was installed in a Beckman DU spectrophotometer equipped with a recording adapter and a Varian G10 recorder. The cuts were made, and the fractions were worked up, as before. The total recovery was 191 g., or 86%.

3. Purification of the Alkaloid Hydrochlorides

Crystalline hydrochlorides were obtained from a majority of the 17 chromatography fractions described above, as listed in Table I. The crystalline products were obtained by dissolving the crude fractions in water, adding various amounts of hydrochloric acid, and letting the solutions cool. In the initial isolation of alkaloid F hydrochloride, crystallization was induced by the addition of a few drops of chloroform to the aqueous solution from which alkaloid G hydrochloride previously had been allowed to crystallize. The total yields of the initial reasonably pure crops of the hydrochlorides obtained directly from the chromatography fractions or from partially purified mixtures are listed in Table II.

The alkaloid hydrochlorides were purified to constant rotation by fractional crystallization from aqueous solutions containing 0.25% to 2.5% hydrochloric acid. The various mother liquors were worked into the crystallization scheme and the compositions of the various fractions were followed by measurements of the specific rotations and the distribution coefficients. The final products and their mother liquors were colorless. The physical properties of the purified crystalline hydrochlorides are listed in Table II.

Table I. Chromatography of Greenheart Alkaloids

Fraction	Eluent Volume, l.[a]	Eluent Conc., %[b]	Yield, %	$[\alpha]_D$, °	K[c]	Crystalline Hydrochlorides Isolated
1	1.9	0.00	6.4	+176	0.12	
2	3.9	0.05	6.1	+210	0.13	
3	5.3	0.09	3.1	+228	0.17	
4	6.7	0.16	2.9	+232	0.19	D
5	8.3	0.31	4.9	+202	0.32	D
6	9.5	0.38	5.4	+127	0.62	D, I, E
7	11.2	0.45	11.3	+69	1.1	E, H, J
8	12.5	0.85	9.5	+41	1.6	E, J, H
9	13.8	1.2	6.7	+62	1.3	E, C
10	14.9	1.8	4.3	+47	1.2	C
11	16.0	2.1	8.3	+68	2.4	G, F
12	17.0	2.6	7.9	+17	3.3	G, F
13	18.3	3.7	5.1	+20	2.5	G, F
14	19.6	5.6	3.0	+47	2.6	F
15	20.9	10	2.3	+47	2.7	
16	22.5	20	2.4	+51	2.8	
17	25.0	50	2.1	+53	2.3	

[a] Average cumulative value for three runs.

[b] Approximate methanol content of the methylene chloride solution at the end of the fraction.

[c] Distribution coefficient in 0.5 $\underline{M}$ acetate buffer, pH 4.17 – chloroform.

Table II. Alkaloid Hydrochlorides From the Chromatography

Hydrochloride of Alkaloid	Yield, %	$[\alpha]_D$ [a]/ °	M. P. [b]/ °	K [c]/
B	[d]/	+250	285	10.5
C	0.9	+74	282	2.3
D	1.8	+74	292	0.72
E	10.7	+97	303	2.8
F	6.5	-181	278	11.5
G	2.7	+236	290	9.1
H	1.0	+268	281	0.39
I	0.8	+148	286	0.50
J	4.2	-38	275	1.4

[a]/ Specific rotation (c 1.0 in water).

[b]/ With decomposition.

[c]/ Distribution coefficient in 0.5 $\underline{M}$ acetate buffer, pH 4.17 - chloroform.

[d]/ Not obtained from the chromatography fractions; entered for comparison.

From the second set of large-scale chromatographies, the hydrochlorides of alkaloids C, D, E, F, and G were obtained in somewhat lower yields than above. The hydrochlorides of alkaloids H, I, and J were not obtained from these chromatography fractions.

J.   Characterization of the Alkaloids

1. Specific Rotations and Melting Points

The purified alkaloid hydrochlorides were initially characterized by their specific rotations and melting points. The specific rotations were obtained with aqueous solutions (c 1.0; i.e., at a concentration of 1 g. per 100 ml.) and are listed in Table II.   The ratios of $[\alpha]_{5461}$ to $[\alpha]_D$ were always approximately 1.22 and were not characteristic of the alkaloid.

The hydrochlorides melted with decomposition. The decomposition points were determined in sealed evacuated capillaries in a type of Hershberg melting-point apparatus at rates of heating of 5° per minute. The decomposition points of different hydrochlorides were not depressed by mixing but were affected by the rate of heating.

Some other melting points were obtained on a Koeffler type of hot stage under a blanket of nitrogen. In these micro melting point (m.m.p.) determinations the crystals on the hot stage were observed under a polarizing microscope.

2. Distribution Coefficients

The distribution coefficients, $\underline{K}$, for the distribution of the alkaloids between the two layers of a 0.5 $\underline{M}$ acetate buffer–chloroform system were determined as follows: A 1-mg. sample of the alkaloid hydrochloride was dissolved in 20 ml. of 0.5 $\underline{M}$ acetate buffer at pH 4.17. This solution was diluted with buffer to give an absorbance reading, $\underline{A}_o$, of about 0.4 in a 1-cm. cell at 283 mμ when compared with the buffer solvent. To a 10-ml. aliquot was added 10 ml. of chloroform (containing 1% alcohol). The resultant mixture was allowed to come to temperature equilibrium in a water bath maintained at 25°, was thoroughly shaken, and was allowed to separate into two layers. The absorbance of the equilibrated buffer solution, $\underline{A}_f$, was determined by comparison with similarly treated buffer solvent. The distribution coefficient was calculated according to the equation:
$\underline{K} = \underline{A}_f / (\underline{A}_o - \underline{A}_f)$.

Values obtained for the various alkaloid hydrochlorides and hydrochloride mixtures at pH 4.17 ranged from about 0.15 to 14. Values for the purified hydrochlorides are listed in Table II.

The distribution coefficients of the hydrochlorides of alkaloids B and G, which were 10.5 and 9.1 in 0.5 $\underline{M}$ acetate buffer, pH 4.17–chloroform, were 1.91 and 1.45, respectively, at pH 5.25. The same alkaloids, in a 0.5 $\underline{M}$ phosphate buffer, pH 6.5–ethyl acetate system, had distribution coefficients of 2.54 and 1.76, respectively. Because the distribution coefficients varied strongly with pH, critical comparisons were always made with the same buffer solutions and were determined simultaneously.

3. Equivalent Weights

In initial runs, 125-mg. quantities of the alkaloid hydrochloride in 35 ml. of water were titrated with one-tenth normal sodium hydroxide in a small beaker. The samples were first dried in vacuum over phosphorus pentoxide. The alkaloids began to precipitate at about pH 7 but were kept well stirred with a Teflon-covered magnetic

stirring bar. The pH was determined with a pH meter and the values were plotted versus the volume of alkali. The midpoint of the inflection of the curve was generally at pH 9.3. Plotting of the rate of change of the pH (ΔpH/Δvolume) versus the pH gave a maximum at the same pH value, and this was taken as the endpoint. From the experimentally determined equivalent weight of the hydrochloride, 36.5 was subtracted to give the equivalent weight of the free alkaloid. The values obtained in duplicate determinations (for alkaloids E, F, G, H, I, and J) agreed within 0.5%; they are listed in Table III.

In later determinations, 50-mg. quantities in 15 ml. of water were titrated in a small cylindrical flask, approximately 30 x 60 mm., under a blanket of nitrogen. The flask was fitted with a stopper containing the two electrodes, a nitrogen inlet tube, and an outlet tube. Through the latter tube was inserted the long thin capillary tip of the burette. When a pH of 8 was reached, the stopper was carefully lifted and the electrodes and sides of the flask were rinsed with water. The titration was completed and the curves were plotted as before. (One duplicate set of determinations without rinsing gave high values averaging 366.5 as compared to an average of 355.5 with rinsing.)

4. Paper Chromatography

4a. Paper Partition Chromatography. — The alkaloids were chromatographed on phosphate-impregnated paper with amyl alcohol, pyridine, water (110:110:90).[33] Whatman No. 1 paper was immersed in 0.2 $\underline{M}$ potassium dihydrogen phosphate, and 15-microgram quantities of the alkaloids were spotted on the paper. The descending development required 14 hours for a total solvent travel of 50 cm.

The best method of developing the chromatogram was to expose the paper to iodine vapors. Iodoplatinate reagent[34] was equally effective when the excess reagent was washed out to reduce the background color. Draggendorff reagent[34] gave only weak spots with the greenheart alkaloids. Folin spray was used in some experiments but was not as effective as the first two methods.

The $\underline{R}_f$ values obtained are listed below, together with the results of the multibuffer paper chromatographies (MBPC) described in Section III.J.4b. The secondary spots produced by alkaloids B and C (listed in parentheses) were quite weak. The secondary spots of alkaloids I and J were quite strong.

Table III. Equivalent Weights of Alkaloid Hydrochlorides

Alkaloid Hydrochloride	Procedure[a]	Equivalent Weight	
		of Hydrochloride	of Free Alkaloid[b]
B	L	335.0	299
D	S	360.8, 360.8	324
E	L	342.0, 343.0	306
E	L	347.5, 344.2	309
F	L	334.5, 333.0	297
G	L	334.5, 334.0	298
G	S	335.3, 335.3	299
H	L	348.5, 350.0	313
I	L	349.5, 350.0	313
J	L	343.5, 344.0	307
O-Methylated E (1)	L	353.2, 353.2	317
O-Methylated E (2)	L	356.1, 356.1	320
O-Methylated E (3)	L	363.4, 363.4	326
O-Methylated E (4)	S	357.1, 354.1	319
O-Methylated G (1)	S	350.9, 352.0	315
O-Methylated G (2)	S	361.1, 360.0	324
O-Methylated G (2)	S	360.4, 360.1	324

a/ L = larger sample, S = smaller sample, as described in the text.

b/ Calculated by subtracting 36.5 from the average values for the
hydrochlorides.

Alkaloid	R_f	MBPC, pH
B	(0.27), 0.33	5.6
C	(0.31), 0.42	5.2
D	0.46	5.0
E	0.40	5.4
F	0.33	5.8
G	0.31	5.6
H	0.34	5.2
I	(0.35), 0.48	5.6, 5.2
J	0.33, (0.43)	5.6, 5.2
Armepavine	0.69	5.2

Other systems tried included butanol, acetic acid, water (63:10:27) on phosphate-impregnated paper,[33] and butanol, acetic acid, water (50:1:50) on untreated paper,[35] but these systems did not give as good a separation of the mixed alkaloids. Preliminary experiments with butanol and buffers[36] at pH 3.0, 5.0, 6.5, and 7.5, with collidine, and with lutidine, gave still less separation. In other preliminary experiments, the quaternary iodides of the mixed alkaloids were chromatographed on paper disks, but several different solvents gave no appreciable separation. The rings of methiodides, which were produced, were developed with a solution of potassium iodate and starch.

4b. Multibuffer Paper Chromatography. — The alkaloids were subjected to multibuffer paper chromatography essentially according to the method of Schmall et al.[37] Strips of Whatman No. 1 paper, 1.5 x 22.5 inches, were ruled at 6 cm. from one end for folding, at 7.5 cm. for spotting, at 9.5 and 11.5 cm. for buffer zone pH 6.4, at 13 and 15 cm. for buffer zone pH 6.2, etc., and at 48 and 50 cm. for buffer zone pH 4.2, leaving 7 cm. of clear paper. The 2-cm. buffer zones were treated with double-strength MacIlvaine buffer solutions which were applied with a large stirring rod. A buffer band of pH 7.6 was applied to the origin of some papers so that alkaloid hydrochlorides could be directly spotted without prior conversion to the free alkaloids.

Fifteen-microgram quantities of the alkaloids were spotted at the origins of the dried papers. After overnight equilibration in the chromatography chamber, the descending chromatographies were run over a period of five hours with chloroform. The paper strips were developed with iodoplatinate reagent, washed with water, dried, and exposed to iodine fumes to further bring out the spots. Initial treatment with iodine fumes was not effective.

The spots were V-shaped, rather than circular, with higher concentrations of alkaloids at the narrow fronts and with wide trailing edges that gradually became weaker as they extended back through two buffer zones. The developed greenheart alkaloid spots were grayish brown and had low intensities. For this reason it was sometimes difficult to distinguish overlapping spots. A sample of armepavine gave a spot considerably darker than those given by the greenheart alkaloids. The pH of farthest advance of each alkaloid is listed in the text-table above.

The ether-soluble alkaloid mixture gave only three main bands, but only one of these bands was in the region of pH 5.4 to 5.8 to which the most abundant alkaloids had advanced. Five samples of alkaloids B and G and their hydrochlorides gave values of 5.8 instead of the values of 5.6 originally obtained. Two samples of O-methylated alkaloid E gave values of 4.8 and 5.0, and three samples of the hydrochlorides of O-methylated alkaloid E gave values of 5.4, 5.6, and 5.6. The reproducibility was poor in one instance where the equilibration period was short. In another instance, and possibly for the same reason, the spots split and ran down the edges of the paper, leaving the center clear.

5. Infrared Spectra

5a. Spectra of Salt Disks. — Infrared spectra of the alkaloid hydrochlorides and of various derivatives were obtained in potassium bromide disks with a Beckmann IR-3 spectrophotometer and, later, with a Beckman IR-7 spectrophotometer. Potassium chloride and potassium bromide disks were originally prepared with a die made according to the design of Ingebrigston and Smith,[38] but with a different sample holder to fit the IR-3. A 5-mg. sample was prepared in 1.5 g. of the potassium salt.

Later samples were prepared with a 1-inch die manufactured by the Applied Research Laboratories, Glendale, California. This die was used in an ARL briquetting machine which developed an 80,000-pound thrust and therefore a pressure of 130,000 pounds per square inch on the sample. Two milligrams of sample in 1.5 g. of potassium bromide were employed.

The sample and the potassium bromide (Harshaw Chemical Co., "Infrared Red Quality") were placed into a stainless-steel capsule containing a 1/4-inch-diameter stainless-steel ball. The contents were mixed in an SS White amalgamator for two minutes. The mixture was poured into the die which was evacuated for five minutes to a pressure of less than 0.1 mm., and the disk was then pressed for five minutes and was removed from the die.

Strong bands were produced at about 2.9μ (3450 cm^{-1}). These bands varied from pellet to pellet, and there was no correlation with various degrees of preliminary drying of the potassium bromide. The bands were stronger when the mixing times of

the samples were increased. Even under the optimum conditions described above, the 2.9μ peak was variable and so large as to mask any potential phenolic peaks in this region.

Some disks were produced by dissolving the sample in 1 ml. of water, adding the potassium bromide in 4 ml. of water, freeze-drying the mixture, and pressing the dehydrated product in the die. This more tedious procedure reduced the 2.9μ peak but did not eliminate it. Drying the potassium bromide overnight at 135°, or the substitution of potassium chloride for the bromide, appeared to reduce the effects of 2.9μ somewhat but did not eliminate them. However, no extensive experiments were carried out, because other regions of the spectra were unaffected and were reproducible.

Some of the spectra which were obtained with the IR-7 spectrophotometer from samples in potassium bromide disks are reproduced in Appendix B.

The spectra of the greenheart alkaloids fell into two groups. The spectra of the alkaloids within each group were very similar to each other, and differences between the spectra of alkaloids not in the same group were much larger.

Into the first group (Group A) fell the spectra of rodiasine dimethochloride and of the hydrochlorides of alkaloids C, D, E, and I, as well as the spectra of derivatives of these alkaloids. The latter derivatives include the hydrochloride and methochloride of O-methylated alkaloid E, free alkaloid E, alkaloid I methochloride, and O-methylrodiasine dimethochloride. The spectra of alkaloid I methochloride and of rodiasine dimethochloride were indistinguishable from each other. The spectra of the hydrochlorides of alkaloids D and I were quite similar and those of the hydrochlorides of alkaloids C and E were also quite similar.

Into the second group (Group B) fell the spectra of the hydrochlorides of alkaloids B, F, G, and H, as well as the spectrum of O-methylated alkaloid G. The spectra of the hydrochlorides of alkaloids B and G were indistinguishable. (The spectrum of alkaloid J had characteristics of both groups.)

The differences between the spectra of the hydrochlorides of alkaloid E and O-methylated alkaloid E were not very great. The spectra of the hydrochloride and the methochloride of O-methylated alkaloid E differed chiefly in the loss of the predominant peaks in the 2400 to 2800 cm^{-1} region. These peaks were also lost in the spectra of the free alkaloids E and G as compared to those of the respective hydrochlorides.

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5b. Spectra of Mulls. — The spectra of alkaloids E and G in hexachlorobutadiene mulls were obtained with the Beckman IR-7. The peaks were weak and were recorded on the 90-100% T scale. The broad band of alkaloid E at 3300 to 3600 cm^{-1} had slight additional peaks at 3550 cm^{-1} and at 3319 cm^{-1}. Alkaloid G gave a comparatively strong band at 3608 cm^{-1} with a shoulder at 3595 cm^{-1}, a much weaker band at 3285 cm^{-1}, and a still weaker band at 3460 cm^{-1}.

5c. Spectra of Solutions. — The spectra of some of the free alkaloids and their acetylated derivatives were determined in chloroform solutions with the Beckman IR-7 from 625 to 4000 cm^{-1}. Solutions of 7.5 mg. of alkaloid in 0.25 ml. of solvent in a cell of 0.2010-mm. path length were employed together with chloroform in a variable path cell in the reference beam. Alkaloids B and F were not sufficiently soluble and their spectra were obtained at lower concentrations.

5d. Near-Infrared Spectra. — Spectra in the 3μ region were obtained with a Beckman DK-2 spectrophotometer. One-centimeter silica cells containing 3 ml. of spectro grade chloroform solution of 2-mg. quantities of the alkaloids were employed. The free alkaloids were freshly prepared from 3-mg. samples of the hydrochlorides by alkalizing the aqueous solutions with ammonia, and by collecting, washing, and drying the precipitates in small, weighed test tubes.

The spectra in the 3μ region again fell into two groups. The spectra of Group A alkaloids (C, D, E, I, and J) had broad peaks at approximately 2.97μ (3365 cm^{-1}), and the spectra of Group B alkaloids (B, F, and G) had sharper peaks at 2.82μ (3545 cm^{-1}). The spectrum of alkaloid H had neither of these peaks but did have a slight peak at 3.03μ (3300 cm^{-1}). The latter peak was barely discernible in the spectra of alkaloids B and G. The positions of the maxima of the spectra of the alkaloids are listed in the table below. The absorbances listed are corrected for concentrations of 2 mg. per 3 ml. of chloroform.

Alkaloid	μ	cm^{-1}	Absorbance
B	2.814	3555	0.16
C	2.972	3365	0.14
D	2.955	3385	0.09
E	2.975	3360	0.12
F	2.820	3545	0.16
G	2.813	3555	0.17
H	3.032	3300	0.03
I	2.92	3380	0.09
J	2.982	3350	0.06

In carbon tetrachloride, alkaloid E gave a peak at 2.93μ (3450 cm^{-1}) instead of the peak at 2.975μ (3360 cm^{-1}) observed in chloroform. Similarly, alkaloid G gave a peak at 2.808μ (3560 cm^{-1}) instead of the peak at 2.813μ (3555 cm^{-1}).

6. Ultraviolet Spectra

Ultraviolet spectra were obtained with a Beckman DK-2 spectrophotometer. Alcoholic or aqueous solutions and 1-cm. silica cells were employed.

Far-ultraviolet spectra, from 230 to 188 mμ, were obtained with 4×10^{-6} $\underline{M}$ aqueous solutions of some of the alkaloid hydrochlorides and quaternary alkaloids. Even though the instrument was purged with nitrogen, stray light became noticeable beyond 198 mμ and reached a 13% transmission reading at 190 mμ and a reading of 37% at 188 mμ. The curves were corrected for stray light by employing the formula $T_c = 100\,(T - T_s)/(100 - T_s)$, where T_c is the corrected percent transmission, T is the observed transmission, and T_s is the transmission reading given by the stray light.

The stray light was determined with dilute aqueous sodium carbonate solution in the sample cell and with water in the reference cell. The sodium carbonate solution transmitted very little light beyond about 225 mμ. The observed T_s readings were not due to light transmission by the sodium carbonate solution at these lower wavelengths, as was shown by the fact that the concentration of the sodium carbonate solutions had no effect on the transmission readings.

The corrected curves showed very broad peaks for all the samples in the range of 205 to 195 mμ. The peaks at 204 mμ of alkaloid E hydrochloride, its O-methylated derivatives, and its O, N-methylated derivative were a little sharper than the very broad 199-mμ peaks of the hydrochlorides of alkaloids B and G. Isochondrodendrine hydrochloride and dimethyltubocurarine chloride had peaks at 202 mμ and 200 mμ, respectively.

The weak 226-mμ peak of dimethyltubocurarine iodide almost disappeared on conversion to the chloride (by treating the solution with excess silver chloride and filtering). Similarly, the differential spectrum of a 10^{-5} $\underline{M}$ solution of this iodide versus 2×10^{-5} $\underline{M}$ potassium iodide gave only a shoulder at 226 mμ, whereas the potassium iodide itself gave a peak at 226 mμ. Isochondrodendrine hydrochloride and tubocurarine chloride gave slight shoulders at 226 mμ which were not observed in the hydrochlorides or methochlorides of the greenheart alkaloids. Alkaloid B methiodide gave a shoulder at 222 mμ, but this disappeared on conversion to the methochloride.

Near-ultraviolet spectra, from 345 to 245 mμ, were observed with 10^{-4} M alcoholic or aqueous solutions. The absorption curves of the greenheart alkaloids and their derivatives began to rise at about 300 mμ and contained maxima at about 281 mμ and minima near 260 mμ, and showed no other chromophoric groups. Similar curves were obtained for isochondrodendrine and tubocurarine chloride and their derivatives. Some maxima and extinction coefficients are listed in Table IV, and some curves are shown in Figures 1, 6, and 7.

Table IV. Ultraviolet Spectra

Compound[a]	Orig. Compound		Phenolate Ion versus Alkaloid			
	λ_{max}, mμ	E[b]	λ_{max}	E	λ_{max}	E
Alkaloid E Hydrochloride	283	9700	313	3600	261	2800
Alkaloid G Hydrochloride	281	9600	302	3600	253	8900
Alkaloid I Methochloride	284	c/	310	c/	254	c/
O-Methylated Alkaloid E Hydrochloride	283	8000	none			
Isochondrodendrine	278	6200	299	9700	256	13,800
Tubocurarine Chloride	282	8900	301	10,800	252	18,200
Phenol	272	3700	290	2900	238	10,800

a/ In 10^{-4} molar alcoholic solution.

b/ E = extinction coefficient = absorbance divided by the concentration.

c/ Concentration uncertain.

The differential spectra of some of the alkaloids versus their hydrochlorides were obtained by filling two cells with alcoholic solutions of the hydrochlorides and adding a drop of 6 N ammonia to the sample cell and a drop of water to the reference cell. Alternatively, a drop of water was added to an alcoholic solution of the free alkaloid in the sample cell and a drop of dilute hydrochloric acid was added to the same solution in the reference cell. Differential peaks (with respective absorbances of 0.37 and 0.21) were obtained for alkaloids E and G at 295 mμ, as

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shown in Figures 6 and 7. Smaller peaks were obtained at 287 mμ and 283 mμ
($\underline{A}$ = 0.13 and 0.11, respectively). Cycleanine gave peaks at 286 mμ and 279 mμ
($\underline{A}$ = 0.16 and 0.14), whereas isochondrodendrine gave much weaker peaks
($\underline{A}$ = 0.06 and 0.03) at the same wavelengths. Similar peaks were obtained with
alcoholic solutions of the hydrochlorides of O-methylated derivatives in the presence
of potassium hydroxide.
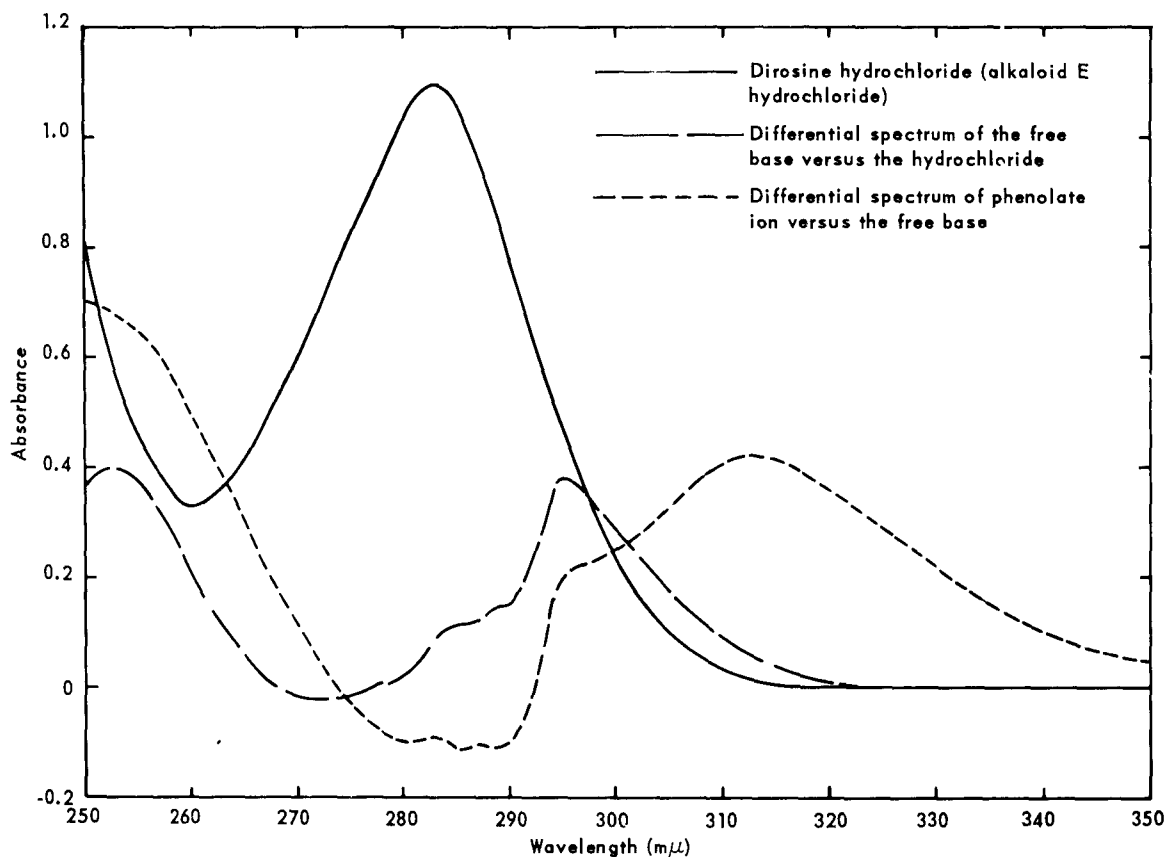
Figure 6. Ultraviolet spectra of dirosine (in 10^{-4} $\underline{M}$ alcoholic
solutions).

Differential spectra of the phenolate ions versus the phenolic alkaloids were obtained by treating the alkaloid solutions in the reference cells with three drops of 20% alcoholic potassium hydroxide and placing three drops of alcohol in the reference solutions. When the original solution was that of an alkaloid hydrochloride, a drop of water in the sample cell and a drop of ammonia in the reference cell were also added so that the differential effect of the change from the hydrochloride to the free alkaloid would not be superimposed on the effect of the potassium hydroxide. Some of the phenolate peaks obtained are listed in Table IV, and two differential spectra are shown in Figures 6 and 7. The hydrochlorides of alkaloids B and F, which are listed in the table, produced differential spectra very similar to that of alkaloid G hydrochloride when sodium hydroxide was added to the aqueous solution. In these spectra the phenolate peaks at about 302 mμ appeared as shoulders superimposed on the 295-mμ differential peaks of the free alkaloids versus the hydrochlorides.
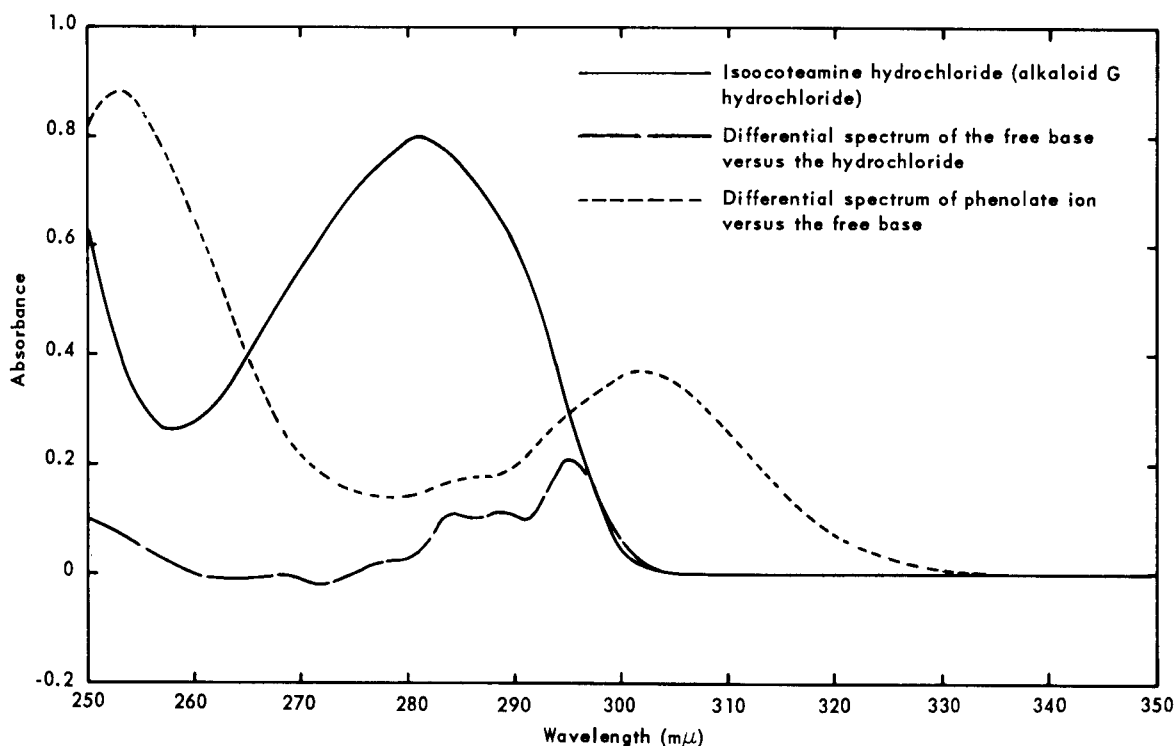
Figure 7. Ultraviolet spectra of isoocoteamine (in 10^{-4} $\underline{M}$ alcoholic solutions).

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No effects were observed when potassium hydroxide was added to alcoholic solutions of completely O-methylated alkaloids. To determine whether small quantities of unreacted alkaloid E in O-methylated alkaloid E could be detected, mixtures of these alkaloids were prepared. The differential absorbances at 313 mμ for 0, 5, 10, and 20% added unreacted material were 0.01, 0.03, 0.045, and 0.08 absorbance units, respectively. O-Methylated alkaloid E hydrochloride (1) gave a 3% absorption beyond the 98% transmission base line at 310 mμ. This absorption is equivalent to approximately 0.01 absorbance units. This weak peak was not present in the spectrum of the O-methylated alkaloid E hydrochloride (2).

7. Tests for Phenolic Groups

7a. <u>Nitroso Derivative and Cobalt Complex Formation.</u> — A small sample of the crystalline material in a test tube was treated with one drop of 5% sodium cobaltinitrite and a drop of glacial acetic acid. The test tube was heated over a free flame, together with another test tube containing only the reagents, until the contents of the latter test tube turned pink.[39] Positive tests, indicated by yellow reaction mixtures, were given by 1-mg. portions of all the alkaloid hydrochlorides, except for alkaloid H hydrochloride, which gave a negative pink color. The hydrochlorides of O-methylated alkaloids E and G also gave negative tests.

7b. <u>Millon's Test.</u> — Into a small test tube was placed 1 mg. of sample and 1/2 ml. of Millon's reagent and the mixture was heated on a steam bath for one minute.[40] Brownish-orange reaction mixtures were considered to be positive tests and yellow reaction mixtures were considered to be negative or indefinite. In most instances precipitates were produced and these were separated by centrifuging and decanting of the solutions and were dissolved in nitric acid. Red solutions so produced were considered to be positive tests. The following results were obtained:

Alkaloid or Hydrochloride	Reaction Mixture	Nitric Acid Solution of the Precipitate
B	+ brownish orange	- yellow
C	- yellow	? orange brown
D	- yellow	- yellow
E	- brownish yellow	+ red brown
F	+ brownish orange	+ red brown
G	+ brownish orange	+ red brown
H	- light yellow	- yellow
I	- brownish yellow	- yellow
J	- yellow	+ red brown
O-Methylated G	- yellow	- yellow
Isochondrodendrine	++ burgundy	(no precipitate)

7c. <u>Folin Test</u>. — The Folin phenol test[28] gave a positive reaction for alkaloid E and a weaker positive reaction for O–methylated alkaloid E. Various attempts to follow the progress of methylation reactions, for example as discussed in Sections III. K. 9f. (1) and (3), were not successful.

8. Acetylation of NH and OH Groups

A 15–mg. portion of the alkaloid or its hydrochloride was dissolved in 0.4 ml. of pyridine and 0.3 ml. of acetic anhydride and the mixture was held at 60° for one hour. The cooled reaction mixture was treated with 4 ml. of water and, after further cooling, was alkalized with ammonia to precipitate the product. The latter was collected by centrifuging, washed twice with water, and dried at reduced pressure.

When the acetylation was performed in the absence of pyridine, alkaloids F and G were completely acetylated as indicated by the absorbance values of 0.41 and 0.43 of the products. However, in the absence of pyridine, alkaloids C, D, and E gave products with absorbances of 0.16, 0.27, and 0.26 for the respective O–acetyl peaks, as opposed to the values listed below for the product obtained by acetylating in the presence of pyridine. (It was also noted that the completely acetylated products of the latter alkaloids precipitated less readily from the alkaline solution than the acetylated alkaloids F or G.)

The infrared spectra of 7.5–mg. portions of the acetylated alkaloids in 0.25–ml. chloroform solutions were determined with a Beckman IR–7 spectrophotometer. A sample cell of 0.2010–mm. path length was employed and a variable–path cell filled with chloroform was kept in the reference beam. The O–acetyl peaks appeared at 1765 cm^{-1} and the N–acetyl peaks at 1630 cm^{-1}. The original alkaloids had much weaker bands at 1610 cm^{-1} and no bands near 1765 cm^{-1}. The absorbances obtained were as follows:

Acetylated Alkaloid	Absorbance at 1765 cm^{-1}	Absorbance at 1630 cm^{-1}
C	0.39	0.62
D	0.40	none
E	0.40	0.63
F	0.41	0.60
G	0.43	0.65
I	0.25	0.40

9. Quaternary Derivatives

Small samples of the hydrochlorides of alkaloids C, D, E, and I were converted to the free bases. The latter were dissolved in methanol and were treated with methyl iodide. The reaction mixtures from alkaloids C and D each deposited a crystalline methiodide which on recrystallization melted at 320° with decomposition. The reaction mixture from alkaloid I also deposited a crystalline methanol-insoluble methiodide. Recrystallization to constant melting point gave a product melting at 314° with decomposition. Alkaloid E did not give a similar methanol-insoluble methiodide.

10. O-Methyl Derivatives

Alkaloid E was prepared from an aqueous solution of the hydrochloride by the addition of ammonia. The resulting precipitate was collected by centrifuging, was washed with water, was dried, and was recrystallized from methanol. Alkaloid E in methanol was treated with an excess of ethereal diazomethane for a period of one day. The reaction mixture was boiled to a small volume and dissolved in methylene chloride. This solution was extracted with 5% aqueous potassium hydroxide and with dilute hydrochloric acid. The hydrochloric acid solution was freed of organic solvent by heating and blowing with nitrogen. The hydrochloride was recrystallized from dilute aqueous hydrochloric acid solutions to constant specific rotation. A portion, converted to the amorphous free base and dissolved in methanol, was again treated with ethereal diazomethane for one day and the product was isolated as above, except that the extraction with potassium hydroxide was omitted. The recrystallized hydrochloride had a $[\alpha]_D$ +107° (c 1.03 in water). It is designated by (2) in Table III. The first hydrochloride crystals are designated by (1); other methylation products are described in Section III. K. 9f.

Alkaloid G was similarly obtained from its hydrochloride. It was O-methylated by treatment of a methanolic solution with ethereal diazomethane for two days. The reaction mixture was evaporated to dryness at reduced pressure, and the product was taken up in very dilute hydrochloric acid and extracted with ether. The hydrochloric acid solution was freed from ether by warming and blowing with nitrogen. After the addition of more hydrochloric acid the product was allowed to crystallize and it was recrystallized to constant rotation; $[\alpha]_D$ +254° (c 1.00 in water). This product is designated by (2) in Table III; other preparations are discussed in Section III. K. 2d.

11. Microanalyses

Initial microanalyses, as reported above, were performed at this Laboratory. Further analytical work was performed at a commercial microanalytical laboratory in Los Angeles which has an excellent reputation. However, considerable difficulty

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was encountered with the alkaloids and reproducible values could not be obtained. Since incomplete combustion and incomplete drying were possible causes for the inconsistencies, extra oxidizer treatment was employed, and the samples were dried to constant weight before each analysis. Even with these extra treatments, consistent results could not be obtained. Values found for six samples of alkaloid E hydrochloride, the last four of which came from the same batch of material and were all subjected to extra oxidizer treatment and dried to constant weight, were as follows:

% C	% H	% CH$_3$O	Dried at
62.21	6.35	12.42	—
61.97	6.51	16.00	—
66.36	6.78	16.39	110°
64.69	6.48	—	80°
63.77	6.57	17.20	80°
65.20	6.60	14.04	80°

Later analyses, including all those listed below, were carried out by Dr. W. Zimmermann at the University of Melbourne, Australia. For two samples of alkaloid E hydrochloride, submitted separately, and dried overnight at room temperature under vacuum and over phosphorus pentoxide, the following values were found:

% C	% H	% CH$_3$O
61.99	6.65	17.33
61.90	6.49	17.23
62.40	6.50	17.34
61.97	6.34	—

Values obtained for the hydrochlorides of the other alkaloids and of two O-methyl derivatives are listed in Table V. Values obtained for alkaloid I hydrochloride and for alkaloid J hydrochloride are omitted, but the values for ocodemarine hydrochloride, subsequently isolated from the latter mixture, are included. (The isolation of ocodemarine is discussed in Section III.K.5b, and the naming of the alkaloids is discussed on page 84.)

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Table V. Microanalytical Results

Alkaloid Hydrochloride (Name & Orig. Desig.)	Empirical Formula	Values	C	H	O	Cl	CH_3O	$N-CH_3$
Group A								
Dirosine, E	$C_{37}H_{42}O_6N_2 \cdot 2HCl \cdot 1\text{-}1/2H_2O$	Calcd.	62.4	6.66	16.9		(4) 17.50	(1) 2.11
		Found	62.0	6.50	16.3		17.30	2.0
Rodiasine, D	$C_{38}H_{44}O_6N_2 \cdot 2HCl \cdot 2\text{-}1/2H_2O$	Calcd.	61.6	6.93	18.3	9.55	(4) 16.75	(2) 4.05
		Found	61.2	6.82	17.8	10.0	16.44	3.8
Norrodiasine, C	$C_{37}H_{42}O_6N_2 \cdot 2HCl \cdot 2H_2O$	Calcd.	61.3	6.73	17.8	9.86	(4) 17.28	(1) 2.1
		Found	61.4	6.51	18.1	10.6	16.95	1.8
Group B								
Ocoteamine, B	$C_{36}H_{38}O_6N_2 \cdot 2HCl \cdot H_2O$	Calcd.	63.1	6.17	16.3		(3) 13.59	(1) 2.19
		Found	62.8	6.19	15.9		13.52	1.6
Isocoteamine, G	$C_{36}H_{38}O_6N_2 \cdot 2HCl \cdot H_2O$	Calcd.	63.1	6.17	16.3		(3) 13.59	(1) 2.19
		Found	63.2	6.29	15.6		13.47	2.2
Otocamine, H	$C_{37}H_{40}O_6N_2 \cdot 2HCl \cdot H_2O$	Calcd.	63.5	6.34	16.0	10.14	(4) 17.78	(1) 2.14
		Found	63.9	6.39	16.8	10.5	17.27	1.9
Demerarine, F	$C_{36}H_{38}O_6N_2 \cdot 2HCl \cdot H_2O$	Calcd.	63.1	6.17	16.3	10.4	(3) 13.59	(1) 2.19
		Found	63.2	6.19	16.8	10.4	13.57	1.9
Ocodemerine, J [g]	$C_{37}H_{40}O_6N_2 \cdot 2HCl \cdot 1\text{-}1/2H_2O$	Calcd.	62.7	6.40	16.9		(4) 17.54	(1) 2.12
		Found	62.8	6.64	16.1		17.60	2.1
O-Methyl Derivatives								
O-Methyldirosine	$C_{38}H_{44}O_6N_2 \cdot 2HCl \cdot 1\text{-}1/2H_2O$	Calcd.	63.0	6.82	16.6		(5) 21.4	
		Found	62.8	6.66	16.5		21.24	
O-Methylisocoteamine	$C_{37}H_{40}O_6N_2 \cdot 2HCl \cdot H_2O$	Calcd.	63.5	6.34	16.0		(4) 17.8	
		Found	63.4	6.34	16.4		17.58	

g/ Originally obtained as a component of this mixture of hydrochlorides.

K. Derivatives and Degradation Products

1. Ocoteamine

1a. Ocoteamine Hydrochloride. — The preparation of ocoteamine hydrochloride, originally designated alkaloid B hydrochloride, by direct crystallization from hydrochloric acid solutions of the ether-soluble alkaloids, (G) and (W), has been described in Section III. G. 1. The distribution coefficient, $\underline{K}$ = 10.5 (in 0.5 $\underline{M}$ acetate buffer, pH 4. 17 – chloroform), 1.91 (1.92 and 1.90 in 0.5 $\underline{M}$ acetate buffer, pH 5.25 – chloroform), and 2.54 (in 0.5 $\underline{M}$ phosphate buffer, pH 6.52 – ethyl acetate). Additional properties are listed in Table IX (page 86), the experimental equivalent weight is shown in Table III, microanalytical results are given in Table V, and the infrared spectrum is reproduced in Appendix B.

1b. Ocoteamine. — An aqueous solution of 0.50 g. of the hydrochloride was treated with ammonia. The resulting precipitate was collected by centrifuging, was washed twice with cold water, and was freeze-dried. The 424 mg. of product was recrystallized from acetone to give 308 mg. of platelets, which were recrystallized again from 10 ml. of methanol to give 240 mg. of rhomboid platelets of ocoteamine. The latter melted at 221.5-222.5° in a sealed capillary.

1c. N-Methylocoteamine Hydrochloride. — A 277-mg. portion of ocoteamine was treated with 1 ml. of 90% formic acid and 1 ml. of 37% formalin and the resulting solution was heated in a steam bath under reflux for four hours. The mixture was kept overnight, was diluted with 20 ml. of water, and was alkalized with ammonia. The resulting precipitate was centrifuged, washed with water, and freeze-dried to give 287 mg. of product. Crystallization from dilute hydrochloric acid gave 227 mg. of fine needles; $[\alpha]_D$ +182°. Recrystallization from dilute hydrochloric acid did not change the specific rotation.

The crystalline hydrochloride was dissolved in water and was converted to the hydrogen sulphate by the addition of a saturated solution of sodium sulphate. The resulting hydrogen sulphate crystals were recrystallized and were then dissolved in water to which hydrochloric acid was added. The first two crops consisted of 64 mg. of long fine needles of $\underline{N}$-methylocoteamine hydrochloride; $[\alpha]_D$ +186° ($\underline{c}$ 1.04 in water); $\underline{K}$ = 2.53 (in 0.5 $\underline{M}$ acetate buffer, pH 4. 15 – chloroform).

1d. O-Methylocoteamine Hydrochloride. — Ethereal diazomethane was prepared as follows:[41] A solution of 43 g. of N-methyl-N-nitroso-p-toluenesulfonamide in 250 ml. of ether was added dropwise over a period of about 45 minutes to a solution of 11.5 g. of 85% potassium hydroxide in 15 ml. of water and 50 ml. of ethanol, kept at 65°. After the addition was completed an additional 30 ml. of ether was added.

The resulting diazomethane and ether were distilled through a water-cooled condenser and were collected in two Erlenmeyer flasks, of 500-ml. and 125-ml. capacities, connected in series. The second of these flasks contained 40 ml. of ether, and both were cooled to -20°. The 280 ml. of distillate contained approximately 6 g. of diazomethane, and was thus approximately 0.5 $\underline{M}$.

A solution of 247 mg. of ocoteamine in 50 ml. of methanol was treated with 30 ml. of the above solution of diazomethane. The solution was kept overnight in a refrigerator and after one day it was evaporated to a volume of 25 ml. and an additional 30 ml. of ethereal diazomethane was added. After a total of two days, the solution was heated to remove the diazomethane and was evaporated to dryness under vacuum. The product was dissolved in a small quantity of very dilute hydrochloric acid. The solution was extracted with ether and then freed of residual ether by warming and flushing the solution with nitrogen. Additional hydrochloric acid was added and the solution was allowed to crystallize. Addition of acid to the mother liquors gave a second crop of crystals, making a total yield of 130 mg. of crude hydrochloride crystals. Recrystallization from 15 ml. of water containing 15 drops of concentrated hydrochloric acid gave 101 mg. of crystals and a second recrystallization gave 83 mg. of O-methylocoteamine hydrochloride. Both products had the same specific rotation; $[\alpha]_D$ +263° (c 1.02 in water).

A second O-methylation of 130 mg. of ocoteamine gave 52 mg. of O-methylocoteamine hydrochloride crystals which were worked up with the mother liquors from the above crystallization and with the solutions from the specific rotation measurements, to give an additional 104 mg. of product; $[\alpha]_D^{26}$ +268° (c 0.50 in water), $[\alpha]_D^{26}$ +279° (c 0.27 in water); $\underline{K}$ = 0.47 (in 0.5 $\underline{M}$ acetate buffer, pH 4.17 - chloroform); $\underline{R}_f$ = 0.62 (for the free alkaloid, see Section III. J. 4a).

<u>Anal.</u> Calcd. for $C_{37}H_{40}O_6N_2 \cdot 2HCl \cdot 1-1/2H_2O$: C, 62.6; H, 6.40; O, 16.9; $4CH_3O$, 17.5. Found: C, 62.9; H, 6.55; O, 17.3; CH_3O, 17.2.

1e. <u>Ocoteamine Methiodide.</u> — A sample of ocoteamine in methanol was treated with excess methyl iodide but gave no methanol-insoluble crystalline product. Two recrystallizations of the reaction product from water gave an amorphous product melting at 252-253°; $[\alpha]_D^{21}$ +51° (c 0.12 in methanol).

2. Isoocoteamine

2a. <u>Isoocoteamine Hydrochloride.</u> — Crystalline isoocoteamine hydrochloride, originally designated alkaloid G hydrochloride, was obtained from chromatography fractions 11, 12, and 13, as indicated in Section III.1.3. Recrystallization to constant specific rotation gave needles; $[\alpha]_D^{22}$ +236° (c 1.023 in water),

$[\alpha]_D^{22}$ +236° ($\underline{c}$ 1.024 in water), $[\alpha]_{5461}^{22}$ +283° ($\underline{c}$ 1.023 in water); m.p. 290° dec.; $\underline{K}$ = 9.1 (in 0.5 $\underline{M}$ acetate buffer, pH 4.17 – chloroform), 1.45 (1.47 and 1.43 in 0.5 $\underline{M}$ acetate buffer, pH 5.25 – chloroform), and 1.76 (in 0.5 $\underline{M}$ phosphate buffer, pH 6.25 – ethyl acetate). Additional properties are listed in Table IX; results of equivalent-weight determinations are shown in Table III; microanalytical results are given in Table V; the ultraviolet spectrum is reproduced in Figure 7; and the infrared spectrum, identical to that of ocoteamine hydrochloride, is reproduced in Appendix B.

2b. <u>Isoocoteamine.</u> — Ammonia was added to a solution of the hydrochloride in water, and the resulting precipitate was collected by centrifuging, washed with water, and freeze-dried. The resulting crude product was readily crystallized from methanol, but could not be readily freed from colored impurities in this manner. Crystallization from acetone gave a colorless product. The latter was recrystallized from benzene and also from methanol. Isoocoteamine from acetone or from benzene melted in a broad range near 180°. Recrystallization from methanol gave rhomboid platelets melting at 219-220°; $[\alpha]_D^{25}$ +392° ($\underline{c}$ 0.29 in chloroform). The infrared spectrum of 7.5 mg. of isoocoteamine in 0.25 ml. of chloroform was somewhat similar to that of oxyacanthine. It was indistinguishable from that of ocoteamine; however, the ocoteamine was not as soluble in chloroform, and the 7.5-mg.-per-ml. solution of the latter alkaloid, which was employed, therefore had weaker peaks.

<u>Anal.</u> Calcd. for $C_{36}H_{38}O_6N_2$: C, 72.7; H, 6.44; O, 16.1; N, 4.71; 4CH_3O, 15.7; 1N-CH_3, 2.5. Found: C, 72.7; H, 6.49; O, 16.7; N, 4.81; CH_3O, 15.4; N-CH_3, 2.3.

2c. <u>N-Methylisoocoteamine Hydrochloride.</u> — A 350-mg. portion of isoocoteamine was dissolved in 1 ml. of 90% formic acid and 1 ml. of 37% formalin and was heated in a steam bath under reflux for three hours. The reaction mixture was dissolved in approximately 20 ml. of water and treated with ammonia. The resulting precipitate was collected by centrifuging, washed three times with water, and freeze-dried to give 332 mg. of crude product. A solution of 7.5 mg. of the product in 0.25 ml. of chloroform gave an infrared absorption curve very similar to that of oxyacanthine. This crude product was dissolved in water containing a small amount of hydrochloric acid and after the addition of 25 drops of saturated sodium sulphate solution to the 13 ml. of solution, a crystalline hydrogen sulphate was obtained. A similar recrystallization gave 248 mg. of crystalline hydrogen sulphate. (A 13-mg. sample of these crystals was acetylated with acetic anhydride in pyridine to give a product which had no detectable band at 1630 cm^{-1}. A Liebermann test[42] for secondary amino groups was inconclusive.)

The hydrogen sulphate was recrystallized from 20 ml. of water containing 18 drops of concentrated hydrochloric acid to give 161 mg. of long needles of the hydrochloride; $[\alpha]_D$ +186° ($\underline{c}$ 0.99 in water); $\underline{K}$ = 2.59 (in 0.5 $\underline{M}$ acetate buffer, pH 4. 15 - chloroform).

2d. O-Methylisoocoteamine Hydrochloride. — A 1.5-g. portion of isoocoteamine in 250 ml. of methanol was treated with 95 ml. of an approximately 0.5 $\underline{M}$ ethereal solution of diazomethane. After one day, the solution was evaporated to 125 ml. and an additional 75 ml. of diazomethane solution was added. After a second day the solvents were removed under reduced pressure. The product was taken up in water to which a little hydrochloric acid was added and the solution was extracted with ether. The solution was freed from ether by heating on a steam bath under a flow of nitrogen and, after the addition of acid to give 80 ml. of 2% hydrochloric acid solution, 951 mg. (a 54% yield) of crude crystalline product was obtained. Two recrystallizations to constant specific rotation gave 685 mg. of long platelets. $[\alpha]_D$ +256° ($\underline{c}$ 1.04 in water), $[\alpha]_D^{26}$ +258° ($\underline{c}$ 0.48 in water); $\underline{K}$ = 0.33 (in 0.5 $\underline{M}$ acetate buffer, pH 4. 17 - chloroform); $\underline{R}_f$ = 0.62 (for the free alkaloid, see Section III. J. 4a).

In a second preparation, a 55% yield of the crude hydrochloride was obtained. In both cases recrystallization from dilute hydrochloric acid gave long flat needles and then smaller rods from the mother liquors; these, however, showed no difference in specific rotation. No differences were detected in the infrared spectra, in potassium bromide pellets, of O-methylisoocoteamine hydrochloride and of O-methylocoteamine hydrochloride.

Anal. Calcd. for $C_{37}H_{40}O_6N_2 \cdot 2HCl \cdot H_2O$: C, 63.5; H, 6.34; O, 16.0; 4CH_3O, 17.8. Found: C, 63.4; H, 6.34; O, 16.4; CH_3O, 17.6.

In the first O-methylation of isoocoteamine, the hydrochloride dissolved in methanol was treated directly with diazomethane. The product so obtained had a slightly higher specific rotation, $[\alpha]_D$ +262° ($\underline{c}$ 1.01 in water), and a somewhat lower equivalent weight. The latter is indicated in Table III, under the listing O-methylated G (1).

2e. O-Methylisoocoteamine. — The free base was obtained by treating an aqueous solution of the hydrochloride with ammonia, and by centrifuging, washing, and drying of the product. Crystallization attempts with a wide variety of solvents were unsuccessful.

A 102-mg. sample of O-methylisoocoteamine was chromatographed on a 1.8 x 5-cm. column of approximately 13 g. of neutral alumina of activity II. The eluents were three 80-ml. portions of benzene and three 80-ml. portions, each,

of 0.25%, 1%, and 5% methanol in benzene. Essentially all the product was eluted in fractions 4, 5, and 6, which contained 33 mg., 62 mg., and 10 mg., respectively. Fraction 6, dissolved in 3 ml. of carbon tetrachloride, showed no peak at 2.81μ (3560 cm^{-1}) in its infrared absorption spectrum. All of fraction 4 dissolved in 3 ml. of carbon tetrachloride likewise gave no discernible peak at 2.81μ. It did give a peak at 3.025μ (3310 cm^{-1}), which was 0.06 absorbance units higher than its base line. Fraction 4 had readily dissolved in the carbon tetrachloride, but soon a precipitate developed. Evaporating to dryness gave 40 mg. of residue, and therefore a gain in weight of 7 mg., or 21%. This residue gave a positive Beilstein test,[43] and microanalyses showed the presence of 20.7% chlorine and 15.6% oxygen.

2f. <u>Reaction With Sodium in Liquid Ammonia.</u> — A solution of 354 mg. of O-methylisoocoteamine hydrochloride was treated with ammonia and extracted with ether. Evaporation of the ethereal solution gave approximately 300 mg. of O-methylisoocoteamine. The latter was reduced with sodium in liquid ammonia essentially according to the method of Section III. K. 6f. After the initial addition of 250 mg. of sodium to produce a lasting blue color, the alkaloid in 10 ml. of benzene –toluene (1:1) was added in seven portions to 150 ml. of liquid ammonia. An additional 250 mg. of sodium was added in 50-mg. portions, to maintain the blue color as the reaction proceeded and for one-half hour after the addition of the alkaloid. The products were treated as described in Section III. K. 6f to give 21 mg. of nonphenolic product and 279 mg. of phenolic product. The latter was dissolved in 2 ml. of ethanol and to this solution was added 2 ml. of a saturated alcoholic solution of oxalic acid. Obtained were 196 mg. of crude oxalate crystals which were recrystallized from ethanol to give 165 mg. of <u>d</u>-armepavine oxalate, m.m.p. 215-216° with effervescence (211-212° uncorrected). Reported values are uncorrected m.p. 211-212° (efferv.),[44] and m.p. 209°.[45]

3. Otocamine

3a. <u>Otocamine Hydrochloride.</u> — This hydrochloride, originally designated alkaloid H hydrochloride, was obtained primarily from fraction 7 of the chromatography, as indicated in Section III. I. 3. Additional quantities were obtained from mixtures with alkaloid D hydrochloride and alkaloid I hydrochloride, as described in Section III. K. 8d. The material was readily recrystallized in the form of small rods which were comparatively easy to handle; $[\alpha]_D^{26}$ +268° (c 1.00 in water), $[\alpha]_{5461}$ +320° (c 1.02 in water); m.p. 281° dec.; $\underline{K}$ = 0.39 (in 0.5 $\underline{M}$ acetate buffer, pH 4.17 – chloroform); $\underline{R}_f$ = 0.62 (for the free alkaloid, see Section III. J. 4a). Additional properties are listed in Table IX; results of equivalent-weight determinations are shown in Table III; microanalytical results are given in Table V; and the infrared spectrum is reproduced in Appendix B.

3b. <u>Otocamine</u>. — A sample of the hydrochloride was dissolved in water and treated with ammonia. The free base was extracted with ether and the ethereal solution was evaporated under reduced pressure. The product could not be crystallized from ether, methanol, or benzene.

3c. <u>N-Methylotocamine Hydrochloride</u>. — A 322-mg. portion of otocamine in 1 ml. of 90% formic acid and 1 ml. of 37% formalin was heated in a steam bath under reflux for four hours. The cooled reaction mixture was dissolved in 20 ml. of water and alkalized with ammonia. The resulting precipitate was collected by centrifuging, washed twice with cold water, and freeze-dried to give 313 mg. of crude product. The latter could not be crystallized from methanol, benzene, or ether, and was dissolved in very dilute hydrochloric acid and extracted with ether. Crystallization from 2% hydrochloric acid gave 223 mg. of needles; $[\alpha]_D^{26}$ +226° (c 0.98 in water), not changed by recrystallization. The infrared spectrum is identical with that of O-methyloxyacanthine hydrochloride.

4. Demerarine

4a. <u>Demerarine Hydrochloride</u>. — Demerarine hydrochloride, originally designated alkaloid F hydrochloride, was obtained from fractions 11, 12, 13, and 14 of the gradient elution chromatography described in Section III.1.2. Addition of chloroform to the mother liquors from the crystallization of isoocoteamine hydrochloride gave a crystalline deposit which was recrystallized to give short hexagonal rods; $[\alpha]_D$ -164°. The latter material, after thorough drying over calcium chloride, lost 2% of its weight after further drying over phosphorus pentoxide for five days, and lost an additional 2% after drying over phosphorus pentoxide at 113°; upon dissolving in warm water, the material gave off the typical odor of a chlorinated solvent. Recrystallization to constant specific rotation from 2.5% hydrochloric acid gave hexagonal platelets of demerarine hydrochloride; $[\alpha]_D$ -181° (c 0.96 in water). Another sample gave $[\alpha]_D$ -176° and $[\alpha]_{5461}$ -213°, a ratio of 1.21; m.p. 278° dec. Additional properties are listed in Table IX; results of equivalent-weight determinations are listed in Table III; and microanalytical results are listed in Table V.

4b. <u>Demerarine</u>. — Initial experiments to produce crystalline demerarine gave comparatively small amounts of the desired products and considerable decomposition products. Thus, ammonia was added to an aqueous solution of 2.5 g. of demerarine hydrochloride, and the resulting precipitate was collected by centrifuging, washed with water, and freeze-dried to yield 2.2 g. of crude demerarine. Crystallization from acetone and methanol gave only 0.45 g. of needles in addition to considerable amber amorphous product.

Subsequently a 1.73-g. portion of demerarine hydrochloride was similarly converted to 1.52 g. of crude demerarine. Crystallization of the latter from methanol gave 1.07 g. of needles plus additional crystals in the mother liquors; m.m.p. 222-223°.

4c. Reaction With Diazomethane. — One gram of demerarine hydrochloride in 10 ml. of methanol was treated with 10 ml. of 0.5 $\underline{M}$ ethereal diazomethane. The yellow color was immediately discharged and the solution was treated with an additional 40 ml. of ethereal diazomethane over a period of two days. The reaction mixture was evaporated to dryness, dissolved in very dilute hydrochloric acid, extracted with ether, and freed of residual ether by warming under a stream of nitrogen. Further acidification to give 45 ml. of 2.5% hydrochloric acid gave a gelatinous product which, after collecting and drying, weighed 415 mg.; $[\alpha]_D^{24}$ -155° ($\underline{c}$ 0.99 in water). Recrystallization again gave gelatinous crystals which on drying weighed 240 mg.; $[\alpha]_D$ -150° ($\underline{c}$ 1.01 in water). Freeze-drying of the mother liquors gave a residue having $[\alpha]_D$ -143° ($\underline{c}$ 0.94 in water).

5. Ocodemerine

5a. Alkaloid J Hydrochloride. — Alkaloid J hydrochloride was obtained from fractions 7 and 8 of the gradient elution chromatography described in Section III.I.2. Recrystallization to constant specific rotation from 1.5% hydrochloric acid gave $[\alpha]_D$ -38° ($\underline{c}$ 1.06 in water); m.p. 175° dec. Additional properties are listed in Table IX, and results of equivalent-weight determinations are listed in Table III.

5b. Ocodemerine Hydrochloride. — A 1.83-g. portion of alkaloid J hydrochloride was recrystallized from 25 ml. of ethanol containing five drops of concentrated hydrochloric acid, to give 0.49 g. of needles having $[\alpha]_D$ +92°, and, on evaporation, 0.16 g. of needles having $[\alpha]_D$ +88°. Careful fractional crystallization of the residue from 2.5% aqueous hydrochloric acid at approximately 50°, to prevent gel formation which occurred at lower temperatures, gave ocodemerine hydrochloride; $[\alpha]_D^{25}$ -170° ($\underline{c}$ 0.99 in water). Analytical results are listed in Table V.

6. Rodiasine

6a. Rodiasine Hydrochloride. — Rodiasine hydrochloride, originally designated alkaloid D hydrochloride, was obtained from fractions 3, 4, and 5 of the counter-current distribution discussed in Section III.H, and it was also obtained from fraction 1 of the conventional chromatography of the ether-soluble alkaloids, as described in Section III.I.1. Larger quantities were obtained from fractions 4, 5, and 6 of the gradient elution chromatography described in Section III.I.2. Recrystallization to constant specific rotation from 1% hydrochloric acid gave rodiasine

hydrochloride; $[\alpha]_D$ +74° (c 1.01 in water); m.p. 291° dec. Additional properties are listed in Table IX; results of equivalent-weight determinations are shown in Table III; microanalytical results are listed in Table V; and the infrared spectrum is reproduced in Appendix B.

6b. <u>Rodiasine</u>. — A 900-mg. portion of rodiasine hydrochloride was dissolved in water and was treated with ammonia. The precipitate was collected by centrifuging, was washed, and was dried to give 761 mg. of product. Crystallization from methanol gave a total yield of 581 mg. of crystals. Recrystallization from methanol did not appreciably change the indefinite m.p. near 176°. The m.m.p. was also quite broad; the crystals lost their original brightness at 158°; they began to fuse at 162°; and the formation of liquid began at 165° and continued to increase very slowly. Recrystallization from ethanol gave very fine needles of rodiasine; $[\alpha]_D^{25}$ +153° (c 0.64 in chloroform); m.p. 209-211°. The m.m.p. was also much sharper; the crystals, originally dark in polarized light, began to show bright areas at 200°; the mass was fused and showed maximum brightness at 205°; liquid and crystals were present at 207°; the crystals almost disappeared at 209°; and they were completely gone at 210°. An m.p. of 195° and $[\alpha]_D^{18}$ +134° (c 0.63 in chloroform) have been reported.[46]

Anal. Calcd. for $C_{38}H_{44}O_6N_2$: C, 73.05; H, 7.10; O, 15.37; N, 4.48; 4CH$_3$O, 19.87. Found: C, 72.29, 71.98; H, 6.73, 6.53; O, 15.3; N, 4.56; CH$_3$O, 19.51.

6c. <u>Rodiasine Dimethiodide</u>. — A 61-mg. sample of amorphous rodiasine, freshly prepared from the hydrochloride, dissolved in 5 ml. of methanol and treated with 1 ml. of methyl iodide, on standing overnight deposited 75 mg. of large cubic crystals. Recrystallization from methanol gave rodiasine dimethiodide; m.p. 321° dec.

Anal. Calcd. for $C_{40}H_{50}O_6N_2I_2$: C, 52.87; H, 5.55; O, 10.6; 4CH$_3$O, 13.66. Found: C, 52.68; H, 5.54; O, 11.0; CH$_3$O, 13.53.

The preparation of other quaternary derivatives of rodiasine has been described in Section III.F.

6d. <u>O-Methylrodiasine Hydrochloride</u>. — One gram of rodiasine in 50 ml. of methanol, kept at 5°, was treated with 80 ml. of approximately 0.5 $\underline{M}$ ethereal diazomethane and with another 50 ml. of this solution on the following day. After a total of two days, the reaction mixture was evaporated to dryness and was dissolved in very dilute hydrochloric acid. The acid solution was extracted with ether, was freed of ether by warming under a stream of nitrogen, and after the further addition of hydrochloric acid was cooled to give crescent-shaped needles.

The mother liquors, further acidified to give 50 ml. of a 2.5% hydrochloric acid solution, produced more crystals giving a total of 870 mg. (an 80% yield). Recrystallization did not essentially change the specific rotation; $[\alpha]_D^{26}$ +54° (c 1.03 in water).

Anal. Calcd. for $C_{39}H_{46}O_6N_2 \cdot 2HCl \cdot H_2O$: C, 64.3; H, 6.91; O, 15.4; $5CH_3O$, 21.3. Found: C, 64.5; H, 6.71; O, 15.3; CH_3O, 21.6.

6e. <u>O-Methylrodiasine</u>. — A 500-mg. portion of O-methylrodiasine hydrochloride was dissolved in water and treated with ammonia. The product was extracted with ether, and the solution was evaporated to dryness to give 411 mg. of the crude free alkaloid. Recrystallization from a small quantity of methanol gave 331 mg. of crystals; m.p. approximately 180°, broad and indefinite. Recrystallization from ethanol or ether was not successful. Recrystallization from methanol, in which the solubility was approximately 30 mg. per ml., gave platelets; $[\alpha]_D^{25}$ +96° (c 0.62 in chloroform). An m.p. of 172-173° (for material crystallized from ether) and $[\alpha]_D$ +85° (c 0.57 in chloroform) have been reported.[46]

Anal. Calcd. for $C_{39}H_{46}O_6N_2$: C, 73.33; H, 7.26. Found: C, 72.51; H, 6.67.

6f. <u>Reaction With Sodium in Liquid Ammonia</u>. — The apparatus for this reaction consisted of a three-necked standard-taper 100-ml. flask. The center neck accommodated an efficient stirrer with a close-fitting oiled sleeve; one neck held a dropper with a rubber bulb and with a standard-taper connection; the other neck was vented through a cold finger and could be opened for the addition of the sodium. The apparatus was initially swept with nitrogen and was placed in a well-stirred bath cooled with dry ice.

Into the flask was poured 75 ml. of liquid ammonia which was held at -35° to -45°. The initial addition of 20 mg. of sodium maintained a blue color for approximately five minutes. A solution of 36 mg. of O-methylrodiasine in 5 ml. of benzene – toluene (1:1) and an additional 180 mg. of sodium were added over a period of one-half hour. One hour after the final addition, the blue color was discharged by adding a total of eight drops of methanol in toluene. (In most of the other reductions, the color was allowed to discharge naturally.) The ammonia was allowed to evaporate overnight and the product was dissolved in water and in ether and additional sodium hydroxide was added.

An extraction scheme similar to that of Tomita and co-workers[47] was employed to isolate the nonphenolic and phenolic products. The original aqueous solution, containing 5% sodium hydroxide, was acidified with hydrochloric acid and was

extracted with ether to remove nonalkaloidal material. The hydrochloric acid solution was alkalized with ammonia and was extracted with ether to obtain the phenolic cleavage product. The original ether solution was extracted with hydrochloric acid, and the resultant acid extract was alkalized with sodium hydroxide and was extracted with ether to give the nonphenolic cleavage product. The ethereal solutions were cooled and kept at 5° overnight, were decanted from the deposited water, and were evaporated to dryness.

Obtained by the above procedure were 300 mg. of nonphenolic product and 19 mg. of phenolic product. Attempted crystallization of the nonphenolic product from ether and hexane was not successful. Crystallization from dilute hydrochloric acid was likewise not successful, nor could O-methylrodiasine hydrochloride be recovered.

Another portion of O-methylrodiasine, 199 mg., was similarly reacted with sodium in liquid ammonia, except that the final addition of methanol was omitted. The products obtained were again predominantly nonphenolic, 94 mg., and very little phenolic product, 13 mg., was obtained.

7. Norrodiasine

7a. Norrodiasine Hydrochloride. — Norrodiasine hydrochloride, originally designated alkaloid C hydrochloride, was obtained as an impurity in the direct crystallization of ocoteamine hydrochloride, as described in Section III.G.2. Larger quantities were obtained from fractions 9 and 10 of the gradient elution chromatography described in Section III.I.2. Recrystallization to constant specific rotation gave needles; $[\alpha]_D$ +74° (c 1.00 in water); m.p. 282° dec. Additional properties of norrodiasine hydrochloride are listed in Table IX, and microanalytical results are given in Table V.

7b. N-Methylnorrodiasine Dimethiodide. — A 100-mg. portion of norrodiasine hydrochloride was converted to 89 mg. of amorphous free alkaloid. A 67-mg. portion of the latter, dissolved in 2 ml. of methanol, readily gave crystals of norrodiasine. These were not collected, but the solution, diluted to 5 ml. with methanol, was treated with 1 ml. of methyl iodide. Overnight, 48 mg. of cubic crystals deposited, and these on recrystallization gave 35 mg. of methiodide; m.p. 318° dec.

8. Isorodiasine

8a. Alkaloid I Hydrochloride. — Alkaloid I hydrochloride was obtained from fraction 6 of the gradient elution chromatography described in Section III.I.2. Recrystallization to constant specific rotation from dilute hydrochloric acid gave

needles and rods; $[\alpha]_D$ +148° (c 1.02 in water); m.p. 291° dec. Additional properties are listed in Table IX and results of equivalent-weight determinations are shown in Table III.

8b.   <u>Alkaloid I Methiodide.</u> — The material recovered from the equivalent-weight titration of 243 mg. of alkaloid I hydrochloride was converted to 214 mg. of amorphous free alkaloid. The latter, dissolved in 10 ml. of methanol and treated with 1 ml. of methyl iodide deposited colorless crystals on standing overnight. The crystals weighed 174 mg. (a 56% yield based on an equivalent weight of 313); m.p. 309° dec. Recrystallization from methanol raised the m.p. to 314° dec, but a second recrystallization did not raise the decomposition point; $[\alpha]_D$ +69° (c 0.153 in water).

8c.   <u>Alkaloid I Methochloride.</u> — An aqueous solution of 138 mg. of alkaloid I methiodide was treated with two 125-mg. portions of silver chloride. The filtered solution was freeze-dried and was crystallized from 2 ml. of water. The mother liquors were acidified with hydrochloric acid to give three crops of crystals totaling 92 mg. Recrystallization from 7 ml. of 10% hydrochloric acid gave needles of alkaloid I methochloride; $[\alpha]_D$ +80° (c 0.99 in water); m.p. 307° dec.

8d.   <u>Fractional Crystallization of Alkaloid I Hydrochloride.</u> — From a chromatography similar to that described in Section III.1.2 and from the fraction corresponding to fraction 6 of the latter chromatography, a product with $[\alpha]_D$ +150° was obtained which appeared similar to alkaloid I hydrochloride. Recrystallization of this material gave 424 mg. of hydrochloride crystals; $[\alpha]_D$ +165°. The latter was dissolved in water and was precipitated by adding potassium hydroxide and alcohol to give 60 ml. of solution containing 10% potassium hydroxide and 15% alcohol, and this mixture was extracted with 60 ml. of benzene. The aqueous potassium hydroxide layers from the extraction scheme were acidified, evaporated to dryness, redissolved in a small quantity of water, and made alkaline with sodium carbonate solution. Only a faint precipitate was obtained. The combined benzene layer, dried with potassium carbonate, filtered, and evaporated to dryness, gave 410 mg. of alkaloid.

The above free alkaloid was again dissolved in 60 ml. of benzene and was extracted with three 15-ml. portions of Claisen alkali (consisting of 41 g. of 85% potassium hydroxide dissolved in 25 ml. of water, and diluted to 100 ml. with methanol). The alkaline extract was neutralized with hydrochloric acid, was made alkaline with potassium carbonate, and was extracted with benzene. The new benzene extract was extracted with hydrochloric acid solution and this solution was made alkaline with ammonia, but only a slight precipitate was obtained. The original benzene solution was washed with water and was extracted with dilute hydrochloric acid. To this extract was added more hydrochloric acid to give 25 ml.

of a 1% hydrochloric acid solution, which deposited a small amount of very fine hairlike needles. Addition of more concentrated hydrochloric acid to produce a 2.5% solution gave additional crystals, which, however, consisted of a mixture of fine needles and small rods. This mixture was collected and recrystallized from 20 ml. of 1% hydrochloric acid. A mixture was again obtained, but heating of this mixture preferentially dissolved finer needles and allowed the collection of the 130 mg. of fine rods. The cooling of the mother liquors gave 194 mg. of fine hairlike needles. The crystallization scheme was continued and the small rods, recrystallized to constant specific rotation, yielded 102 mg.; $[\alpha]_D^{26}$ +268° ($\underline{c}$ 1.00 in water). The needles were similarly recrystallized to give 131 mg.; $[\alpha]_D^{26}$ +77° ($\underline{c}$ 1.01 in water).

A 50-mg. sample of the above needles was converted to 45 mg. of free base. The latter was dissolved in 8 ml. of methanol and was treated with 1 ml. of methyl iodide. The next morning the solution was evaporated to 6 ml. and was allowed to cool. Slightly yellow cubic crystals, weighing 56 mg., were obtained, and on recrystallization from 6 ml. of methanol, these gave 41 mg. of almost colorless crystals; m.p. 321° dec.

An available 133 mg. of the original alkaloid I hydrochloride was crystallized from 8 ml. of 1% hydrochloric acid. A mixture of the characteristic hairlike needles and of the long rods was obtained. Warming of the solution dissolved the fine needles, and the rods were collected. The careful crystallization, described above, was repeated to give additional rods and needles. Two recrystallizations of the 62 mg. of fine needles produced essentially no change in specific rotation; $[\alpha]_D^{25}$ +73° ($\underline{c}$ 1.01 in water); $\underline{K}$ = 0.59 (in 0.5 $\underline{M}$ acetate buffer, pH 4.17 - chloroform, in which a sample of freshly recrystallized rodiasine hydrochloride concurrently gave $\underline{K}$ = 0.56).

9. Dirosine

9a. Dirosine Hydrochloride. — Dirosine hydrochloride, originally designated alkaloid E hydrochloride, was obtained from fractions 2 to 5 of the conventional chromatography of the ether-soluble alkaloids, as described in Section III.I.1 and shown in Figure 4, and also from fractions 6, 7, and 8 of the countercurrent distribution discussed in Section III.H. Larger quantities were obtained from fractions 6, 7, 8, and 9 of the gradient elution chromatography described in Section III.I.2. Dirosine hydrochloride was readily recrystallized from very dilute hydrochloric acid; $\lceil\alpha\rceil_D$ +97°, $[\alpha]_{5461}$ +114° ($\underline{c}$ 1.02 in water); m.p. 303° dec. The solubility in cold water was about 1.2 g. per 100 ml., in 2% hydrochloric acid it was about 0.25 g. per 100 ml. The loss on drying at reduced pressure over phosphorus pentoxide for three days at room temperature was 5.3%; additional loss on drying at 110° for 4-1/2 hours was negligible. Additional properties are listed in Table IX;

results of equivalent-weight determinations are shown in Table III; microanalytical results are given in Table V; the ultraviolet spectrum is reproduced in Figure 6; and the infrared spectrum is reproduced in Appendix B.

9b. <u>Dirosine</u>. — One gram of dirosine hydrochloride was dissolved in water, ammonia was added, and the precipitate was collected by centrifuging, washed with water, and dried to give 0.89 g. of product. Recrystallization from 50 ml. of methanol gave 0.72 g. of crystals. On heating, these began to fuse at 170°, they produced a clear mass clinging to the side of the capillary at 173°, and they finally began to flow at 194°. Recrystallization from ethanol did not reduce the melting range appreciably.

A 54-plate countercurrent distribution of dirosine was run as described in Section III. H. The system employed was 0.5 $\underline{M}$ acetate buffer, pH 4.79 - chloroform; tube 0 initially contained dirosine in the buffer layer at a concentration of 0.4 mg. per ml. The curve obtained is shown in Figure 8.
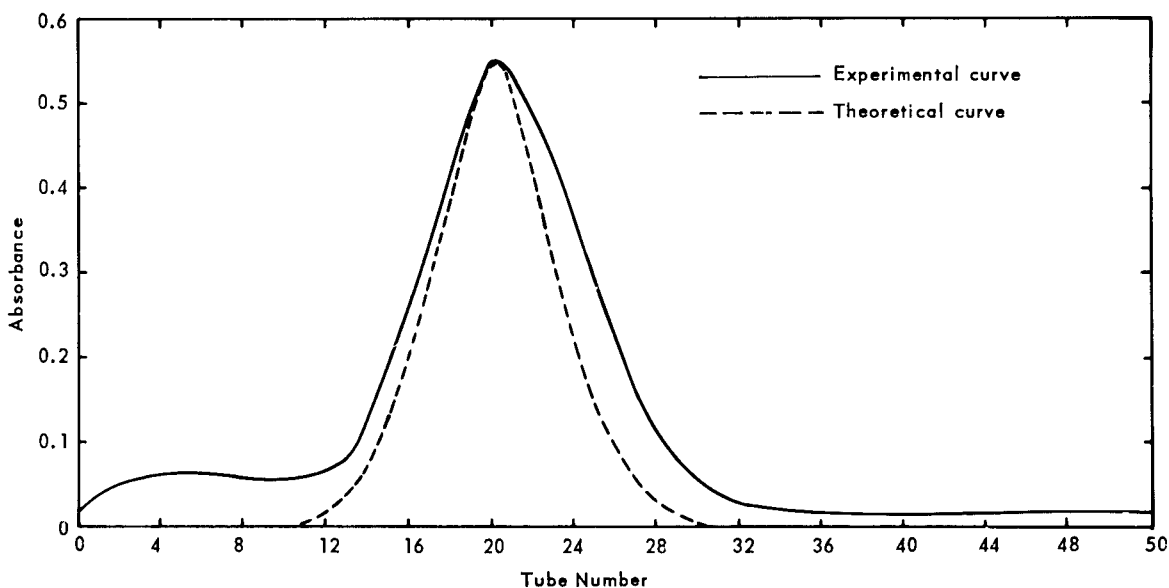

Figure 8. Countercurrent distribution of dirosine.

Anal. Calcd. for $C_{37}H_{42}O_6N_2$: C, 72.76; H, 6.93; O, 15.7; N, 4.59; $4CH_3O$, 20.4. Found: C, 72.9; H, 6.70; O, 16.0; N, 4.36; CH_3O, 19.84.

9c. **Dirosine Methiodide.** — Dirosine in methanol was treated with methyl iodide and was refluxed for two hours. The product, recrystallized from methanol and water, had $[\alpha]_D$ +90° ($\underline{c}$ 1.0 in water); m.p. 290° dec. Another portion recrystallized from methanol and 2-propanol gave colorless needles having $[\alpha]_D$ +99°; all other products in the fractional crystallization were amorphous. Another preparation gave a fraction with $[\alpha]_D$ +103°, and a fraction with $[\alpha]_D$ +82.5°.

9d. **O, N-Dimethyldirosine Dimethiodide.** — A 400-mg. portion of dirosine dissolved in 30 ml. of methanol was treated with 0.3 ml. of methyl iodide and was refluxed for one-half hour. An additional 0.3 ml. of methyl iodide and 3 ml. of 0.5 $\underline{N}$ methanolic potassium hydroxide were added and the solution was refluxed for one hour. The addition of methyl iodide and potassium hydroxide and the refluxing were repeated to make a total of five treatments. Folin phenol tests of three-drop samples of the solution gave decreases in blue color after each of the first four treatments. The evaporated reaction mixture was dissolved in 50 ml. of hot water. The non-crystalline precipitate formed on cooling was collected and washed with a small amount of ice water. The dried product weighed 468 mg. (a 77% yield). Recrystallization from methanol and 2-propanol gave a slightly yellowish amorphous product; $[\alpha]_D^{23}$ +109° ($\underline{c}$ 0.53 in water); m.p. 264° dec. The latter value did not change on recrystallization.

9e. **O, N-Dimethyldirosine Dimethochloride.** — A 994-mg. portion of the above methiodide, dissolved in 100 ml. of water, was treated with three 500-mg. portions of silver chloride. The solution was filtered and freeze-dried to give 714 mg. of crude product (a 94% yield based on the methiodide and a 68% yield based on the original dirosine employed). Recrystallization to constant specific rotation from methanol-acetone gave needles with $[\alpha]_D^{23}$ +136° ($\underline{c}$ 0.7 in water); m.p. 245° dec. Dimethyldirosine dimethochloride lost 7% of its weight on drying four hours over phosphorus pentoxide at 80° or at 110°. The product did not give very reproducible analytical values. The microanalyses which gave the best duplicate values gave the following results.

Anal. Calcd. for $C_{41}H_{52}O_6N_2Cl_2 \cdot 2H_2O$: C, 63.5; H, 7.26; O, 16.5; Cl, 9.15; $5CH_3O$, 20.0. Found: C, 63.91, 63.57; H, 6.81, 7.06; O, 14.7; Cl, 9.45; CH_3O, 19.0.

9f. O-Methyldirosine Hydrochloride. — In addition to two preliminary experiments, seven methylations of dirosine were run. An approximately 0.5 $\underline{M}$ ethereal diazomethane solution, prepared as described in Section III.K.1d, was employed. The experimental conditions for the seven runs were as follows:

(1) A 3.4-g. portion of dirosine hydrochloride was converted to the free alkaloid by treatment with ammonia. The collected, washed, and dried product weighed 2.81 g. Of the latter, 2.65 g. were dissolved in 300 ml. of methanol and treated with 200 ml. of ethereal diazomethane. After one day the mixture was evaporated to a small volume, methylene chloride was added, and the solution was extracted with 5% aqueous potassium hydroxide. The alkaline extract yielded no alkaloids on neutralization, and thus no unreacted dirosine. The methylene chloride solution was extracted with very dilute hydrochloric acid, and the acidic solution was warmed under a stream of nitrogen to expel methylene chloride and was freeze-dried to give 2.04 g. of product. Recrystallization from 80 ml. of 1.5% hydrochloric acid gave fine needles of O-methyldirosine hydrochloride (1) weighing 1.46 g. (a 54% yield); $[\alpha]_D$ +106°, $[\alpha]_{5461}$ +129° ($\underline{c}$ 1.00 in water); m.p. 277° dec.

The above hydrochloride gave an equivalent weight of 317 for the free alkaloid, as shown in Table III. Folin tests with the product, and with the product plus approximately 10% dirosine hydrochloride, gave absorbance readings of 0.47 and 0.85, respectively, at 600 mμ. Ion exchange chromatography on the hydroxyl form of Dowex 1-X_8 and elution with ethanol gave a recovery of 65% based on absorbance measurements of the solutions, but the product still gave a somewhat positive Folin test.

A 313-mg. portion of hydrochloride (1) dissolved in 35 ml. of water plus 24 drops of concentrated hydrochloric acid gave 226 mg. of fine needles, 14 mg. of a mixture of needles and of the characteristic platelets obtained below, and then again a mixture of needles and platelets which was not collected.

(2) A portion of O-methyldirosine hydrochloride (1) was converted to the free alkaloid and dried. An 876-mg. portion of the latter was dissolved in 30 ml. of methanol and was treated with 90 ml. of ethereal diazomethane. After one day, an evaporated sample of the reaction mixture still gave a positive Folin test. The partially evaporated reaction mixture was dissolved in methylene chloride and was extracted with dilute hydrochloric acid. Evaporation of the methylene chloride from the aqueous extract, addition of hydrochloric acid, and cooling, gave 685 mg. of rhomboid platelets of O-methyldirosine hydrochloride (2); $[\alpha]_D$ +107° ($\underline{c}$ 1.03 in water).

A 359-mg. portion of hydrochloride (2) dissolved in 40 ml. of water plus 29 drops of concentrated hydrochloric acid gave 273 mg. of the characteristic platelets obtained above. On the addition of more concentrated hydrochloric acid to the mother liquors, 7-mg., 24-mg., and 12-mg. portions of additional platelets were obtained, but no needles.

(3) Four 1.00-g. portions of dirosine were dissolved in two 100-ml. portions of methanol and in two 50-ml. portions of methylene chloride. A 70-ml. portion of ethereal diazomethane was added to each of the methanol solutions and a 25-ml. portion was added to each of the methylene chloride solutions. One solution of each of the sets was kept at room temperature and a duplicate reaction mixture was kept at 5°. The reaction was continued for ten days and it was intended to follow the course of the reaction with the Folin test and the o-nitrosophenol test. After one day the latter test, described in Section III.J.7a, gave positive results for all reaction mixtures and after four days it gave weak results for the methanol solutions and positive results for the methylene chloride solutions. The Folin test was strongly positive for all solutions after one day but gave somewhat erratic results in subsequent tests. The methanol solutions lost the diazomethane color more rapidly than the methylene chloride solutions, and the latter solutions gave a yellower reaction mixture.

The four reaction mixtures were combined and evaporated to dryness. The product was chromatographed on a 3.4 x 15-cm. column of 150 g. of Woehlm neutral alumina deactivated by 3.38 g. of water. The material was added in methylene chloride solution and a gradient eluent of methanol in methylene chloride was employed. Two major peaks containing 1.31 g. and 3.09 g. of product were obtained. The product from the first peak was not completely soluble in dilute hydrochloric acid and the mixture produced was extracted with methylene chloride to give 466 mg. of neutral material. From the extracted aqueous solution, 463 mg. (a 10% yield) of crystalline hydrochloride (3) were obtained. Recrystallization gave the characteristic platelets of O-methyldirosine hydrochloride (2); $[\alpha]_D$ +107° (c 1.03 in water).

The second and major portion from the chromatography was treated again with diazomethane and the new product, weighing 3.18 g., was again chromatographed. A first peak giving 0.42 g. and a second major peak giving 2.53 g. of product were obtained. No crystalline hydrochlorides were obtained from these products.

Portions of the 466 mg. of neutral material from the first peak of the first of the above chromatographies were subjected to catalytic hydrogenation. Platinum catalyst and ethanol or glatial acetic acid were employed in a modified micro-hydrogenation apparatus.[48, 49] The calculated hydrogen uptake[50] in all instances was considerably less than one mole, based on the molecular weight of dirosine, and no crystalline products were obtained.

(4) One gram of dirosine hydrochloride dissolved in 30 ml. of methanol was kept at room temperature and was treated throughout the day with small portions of ethereal diazomethane solution totaling 110 ml. The solution was kept at 5° overnight and was evaporated to dryness in a water bath at 50° under a stream of nitrogen. The product was dissolved in methylene chloride and the solution was extracted with very dilute hydrochloric acid. The methylene chloride solutions from the extraction, on evaporation, gave 120 mg. of residue. The hydrochloric acid solutions, after removal of methylene chloride and addition of more hydrochloric acid, gave 576 mg. (a 54% yield) of crystals.

Recrystallization from 20 ml. of 2.5% hydrochloric acid gave a mixture of needles, platelets, and amorphous product. Careful recrystallization from 60 ml. of 1.5% hydrochloric acid gave the 334 mg. of the characteristic platelets of hydrochloride (2); $[\alpha]_D$ +103° ($\underline{c}$ 0.98 in water).

(5) A 2.02-g. portion of dirosine hydrochloride in 20 ml. of methanol was treated with 80 ml. of ethereal diazomethane and the following day an additional 100 ml. of diazomethane solution was added. After a two-day reaction period, the unreacted diazomethane was removed by distillation. The resulting solution on dilution with ether gave a precipitate. The ether was removed by distillation and the products were evaporated to dryness in a water bath at 50° under a stream of nitrogen. The products were dissolved in very dilute hydrochloric acid and the resulting aqueous solution was extracted with ether, freed of excess ether under a flow of nitrogen, and was further acidified to give 80 ml. of a 1% hydrochloric acid solution. Obtained were 686 mg. of hydrochloride crystals and, on addition of more hydrochloric acid, an additional 105 mg. of crystals (a total yield of 40%) were obtained. The 686 mg. of crystals were recrystallized from dilute hydrochloric acid to give 410 mg. of O-methyldirosine hydrochloride (5) crystals having $[\alpha]_D$ +101° and $[\alpha]_{5461}$ +122° ($\underline{c}$ 1.00 in water), and a second crop of crystals weighing 143 mg. and having $[\alpha]_D$ +104° ($\underline{c}$ 1.00 in water).

(6) Three grams of dirosine in 300 ml. of methanol were treated with a total of 190 ml. of ethereal diazomethane over a period of two days. The reaction mixtures were kept at approximately 5° during most of this time. After the two-day reaction period, excess diazomethane was removed by distillation and the solution was evaporated. During this evaporation, some free alkaloids crystallized from the solution after seeding with the free base from methylation product (2), m.p. 155°, but these crystals were not separated. The dried reaction mixture was dissolved in dilute hydrochloric acid and the resulting solution was extracted with ether, freed from excess ether, and further acidified and allowed to crystallize. Obtained were 2.09 g. of O-methyldirosine hydrochloride (6) crystals. Three recrystallizations gave flat needles rather than the characteristic platelets of O-methyldirosine

hydrochloride (2) with which the solution was seeded; $[\alpha]_D$ +105° (c 1.00 in water). Small amounts of the characteristic platelets were obtained from the mother liquors of the crystallization scheme employed.

(7) A 1.59-g. portion of the above hydrochloride (6) was converted to the free alkaloid. The latter, in 50 ml. of methanol at 5°, was treated during the day with portions of ethereal diazomethane totaling 110 ml. The solution was kept overnight and was then evaporated to remove most of the ether. During the second day a total of 150 ml. of diazomethane solution was added and the yellow solution was kept at room temperature overnight. After a total reaction time of two days the solution was evaporated and the product was dissolved in hydrochloric acid, extracted with ether, and crystallized as before. Obtained were a total of 0.81 g. (a 50% yield) of O-methyldirosine hydrochloride (7) crystals, which on recrystallization and seeding with the characteristic platelets of O-methyldirosine hydrochloride (2) did not give these platelets but gave large, irregular, layered crystals.

9g. O-Methyldirosine. — The free alkaloids were prepared from a number of the O-methyldirosine hydrochloride products discussed in the preceding section. For ease of reference, the preparations listed below are numbered to correspond with the starting materials obtained above.

(2) A 423-mg. portion of hydrochloride platelets (2) was converted to 375 mg. of dried free alkaloid. Recrystallization from methanol gave 299 mg. of rosettes of fine needles of O-methyldirosine (2B); m.m.p. 156°. Recrystallization from methanol did not change the melting point. The crystals were originally dark in polarized light, began to show bright spots at 140°, reached maximum brightness and began to wet the cover glass at 152°, and lost most of their brightness at 156°.

Forty-five milligrams of the residue from the crystallization of O-methyldirosine (2B) were dissolved in hydrochloric acid. Crystallization gave 46 mg. of the characteristic platelets of O-methyldirosine hydrochloride (2). Similarly, 30 mg. of the crystals (2B) were dissolved in hydrochloric acid and 31 mg. of the characteristic platelets were obtained from the solution.

(4) A 217-mg. sample of O-methyldirosine hydrochloride (4) was dissolved in water and was converted to the free alkaloid by addition of ammonia. The precipitated alkaloid was centrifuged, washed with water, and dried to give 190 mg. of product. Crystallization from 10 ml. of methanol gave 94 mg. of well-defined needles of O-methyldirosine (4B). Evaporation of the mother liquors to 3 ml. gave an additional 36 mg. of needles.

An additional 250 mg. of hydrochloride crystals from the fractional crystallization of O-methyldirosine hydrochloride (4) was converted to 197 mg. of free alkaloid. The latter was chromatographed on a 1.8 x 9-cm. column of 24 g. of neutral alumina of activity II employing, as eluent, mixtures of ether containing small amounts of methanol. The chromatography curve gave four peaks. The third peak eluted with 2% methanol in ether gave 52 mg. of product which on standing gave colorless needles; m.p. 148°. Attempts to recrystallize this product from methanol and successively from acetone, benzene, and ether were not successful; the crystals could not be obtained again.

(5) A 410-mg. portion of O-methyldirosine hydrochloride (5) was converted to 328 mg. of free alkaloid. Crystallization from methanol gave 104 mg. of large flat needles designated (5B), which were similar in appearance to (4B). From the mother liquors an additional 146 mg. of crystals in the form of rosettes was obtained, which on recrystallization gave 100 mg. of rosettes, designated (5C). The m.m.p. of (5B) was 149°. The crystals were originally bright under polarized light but lost their brightness at approximately 140°. The rate of heating was 2° per minute. The m.m.p. of (5C) was 173°. These crystals were dark in polarized light at the beginning of the determination; bright areas began to appear at approximately 157°; both liquid and bright crystals were present at 165°; and the liquid became completely dark at approximately 173°.

A mixed m.m.p. of (5B) with (2B) and a mixed m.m.p. of (5C) with (2B) both gave no depressions and showed the characteristics of both components. In the first of these, the brightness of the needles of (5B) disappeared near 140° and some definite fusing occurred at 149°. The mixture had become bright again near 147° and this brightness increased and then almost disappeared at 154°. In the second mixed m.m.p. the crystals were completely dark at the start of the determination and began to brighten at 142°. The brightness increased and then decreased again in the region of 149-153°. The bright areas increased again at 156°; a liquid mass containing bright crystals was present at 163°; and the brightness disappeared again at approximately 170°.

(7) A 712-mg. sample of O-methyldirosine hydrochloride (7) was converted to 555 mg. of the free alkaloid. Two recrystallizations from 15-ml. portions of methanol gave 199 mg. of rosettes of methylated dirosine (7B); m.m.p. 175°. The crystals were opaque in polarized light at the beginning of the determination; bright areas appeared at 158°; maximum brightness and some wetting occurred at 169°; a decrease in brightness was apparent at 172°; most of the brightness disappeared by 175°; and it was essentially gone at 176°.

For comparison, additional O-methyldirosine (2B) was prepared from the corresponding hydrochloride (2). Two recrystallizations gave needles which in appearance were indistinguishable from the O-methylated dirosine (7B) described above; m.m.p. 176°. The crystals (2B) were opaque in polarized light at the beginning of the determinations; bright areas appeared at 145°; maximum brightness was reached at 153°; liquid and considerably decreased brightness were apparent at 156°; and the brightness had essentially disappeared at 158°. The solutions from which the crystals (7B) and (2B) were obtained had each been seeded with the other alkaloid in order to determine whether different crystalline modifications were involved.

Anal. The following are the values calculated for $C_{38}H_{44}O_6N_2$, with five methoxyl groups and one N-methyl group, and the values found for the products indicated:

Analysis	Calcd.	(2B)	(5B)	(7B)
% C	73.0	71.9	72.5	72.2, 71.4
% H	7.10	6.72	6.96	6.81, 6.45
% O	15.4	—	15.3	16.2
% N	4.48	—	4.48	5.35
% CH$_3$O	24.8	24.1, 24.1	24.2	23.8, 23.8
% N-CH$_3$	2.41	3.3	2.6	3.1

9h. <u>Reaction With Sodium in Liquid Ammonia.</u> — Three reductions of methylated dirosine with sodium in liquid ammonia were carried out. The preparations listed below are numbered to correspond with the designations for the starting materials, Section III. K. 9f.

(2) A 115-mg. portion of O-methyldirosine (2B) was dissolved in 2 ml. of benzene and 2 ml. of toluene. This solution was slowly added, together with small portions of sodium, to 75 ml. of well-stirred liquid ammonia kept at –40° to –45°, in the apparatus described in Section III. K. 6f. To obtain a lasting blue color 100 mg. of sodium was required. An additional 120 mg. of sodium was required to maintain the blue color as the sample was slowly added and to maintain the color for 40 minutes after the final addition. At this time the color was discharged with ammonium chloride.

The crude product was taken up in water and in ether and additional sodium hydroxide was added so that the products could be distributed between ether and approximately 5% sodium hydroxide. The products were worked up as described in Section III. K. 6f to give 56 mg. of nonphenolic product and 33 mg. of phenolic product.

Both of the above products gave positive o-nitrosophenol tests, whereas the starting material had given a negative test. The infrared spectra of the "nonphenolic" and "phenolic" products in chloroform gave peaks at 3525 cm^{-1} and 3552 cm^{-1}, respectively. Paper chromatography with system (A) (pyridine, amyl alcohol, water), as described in Section III. J. 4, gave long streaks for both products.

(4) A similar reduction had been carried out with 82 mg. of methylated dirosine (4B). The ethereal solutions of the product had been dried over anhydrous potassium carbonate as originally suggested by Tomita and co-workers.[45] The nonphenolic and phenolic products weighed only 18 mg. and 19 mg., respectively, and it was evident that considerable material had been absorbed in the drying agent. Both products gave positive o-nitrosophenol tests.

Another reduction of 55 mg. of (4B) was carried out in refluxing liquid ammonia. In the extraction of the products, methylene chloride was substituted for the ether. The 36 mg. of nonphenolic product and the 12 mg. of phenolic product both gave positive o-nitrosophenol tests and weak Millon's tests, whereas the starting material gave negative results in both tests. Beilstein tests[43] were negative for both products.

10. Ether-Soluble Alkaloid Mixtures

10a. O-Methylation. — Seven grams of ether-soluble alkaloids (GW2) in 140 ml. of methanol were treated with 140 ml. of 0.5 $\underline{M}$ ethereal diazomethane at 5° for one day. An aliquot of the reaction mixture gave a yellow distillate. On the second and third days, additional 70-ml. portions of ethereal diazomethane were added and the reaction was allowed to proceed for a total of five days. Evaporation to dryness gave 7.64 g. of product. A chloroform solution of a sample of the latter showed no peaks at 3370 cm^{-1} or 3550 cm^{-1}.

The product was treated with zinc and hydrochloric acid to reduce any amine oxides that might have been formed. Considerable difficulty was encountered by the formation of a precipitate on the addition of the zinc dust. This precipitate was not soluble even after considerable dilution with water and appeared to be less soluble in dilute sulphuric acid. The precipitate was dissolved in 1% hydrochloric acid and the treatment with zinc dust was continued. After several hours the solutions were filtered and were made strongly alkaline with potassium hydroxide solution. Sticky

gums were formed, which were not soluble in ether. The precipitates were collected, dissolved in dilute hydrochloric acid, precipitated with ammonia, collected by centrifuging, and were freeze-dried to give 6.62 g. of product. Attempts to obtain crystalline salts were not successful. Hydrochlorides, bromides, iodides, and nitrates were obtained as amorphous products from aqueous solutions. The addition of tartaric acid or oxalic acid to hydrochloric acid solutions gave no precipitates.

A 5.2-g. portion of the methylated alkaloids was chromatographed on 141 g. of alumina with a gradient eluent consisting of methylene chloride containing increasing amounts of methanol. Five peaks were obtained in the chromatography curve. The products of the fractions represented by the peaks of the curve were dissolved in dilute hydrochloric acid, but even on seeding with O-methylisoocoteamine hydrochloride and O-methyldirosine hydrochloride no crystals were obtained.

A second portion of 19.3 g. of ether-soluble alkaloids was methylated with diazomethane for ten days to give 21.3 g. of product; $[\alpha]_D$ +78° (c 0.97 in $\underline{N}$/10 hydrochloric acid). A 9-g. portion was chromatographed on 210 g. of alumina of activity I with 7.5 liters of gradient eluent consisting of ethyl acetate containing increasing amounts of methanol. The first of nine fractions, eluted with ethyl acetate, contained 35% of the product and had $[\alpha]_D$ +107°. A second major peak was obtained, and in this were included fractions 6 and 7, 11% and 12% of the products, respectively; $[\alpha]_D$ +29° and +38°. Amorphous hydrochlorides were obtained from these fractions. Some months later, the amorphous products from fractions 6 and 7 became partly crystalline.

A third methylation of 19.5 g. of ether-soluble alkaloids with ethereal diazomethane over a period of two days gave a product which was evaporated to dryness, dissolved in dilute hydrochloric acid, extracted with ether, reprecipitated with ammonia, collected by centrifuging, and freeze-dried to give 19.9 g. of O-methylated alkaloids; $[\alpha]_D$ +93° (c 1.03 in $\underline{N}$/10 hydrochloric acid).

10b. O,N-Methylation. — A 10-g. sample of the above product in 20 ml. of 90% formic acid and 20 ml. of formalin was refluxed for eight hours. After the addition of 25 ml. of 5% hydrochloric acid, the solution was evaporated in vacuo. The residue, dissolved in water, was treated with ammonia, and the precipitate produced was collected by centrifuging, washed, and freeze-dried to give 9.7 g. of product, $[\alpha]_D$ +33° (c 1.08 in $\underline{N}$/10 hydrochloric acid).

A 9.1-g. portion of the above product was extracted with 400 ml. of hot benzene to give 2.1 g. of residue. The benzene solution was chromatographed on a 200-g. column of alumina of activity halfway between I and II with a gradient eluent consisting of benzene containing increasing amounts of methanol. Only one major peak was obtained near the center of the chromatogram at a point where the methanol concentration was approximately 0.3%.

L. Alkaloids From Other Sources

1. Isochondrodendrine

1a. Isochondrodendrine. — A sample of commercial Bebeerine Hydrochloride (T. & H. Smith, Ltd., Scotland) was converted to the free alkaloid by treatment with aqueous sodium carbonate, but the product obtained did not have the properties of "bebeerine." The manufacturer, after being questioned, stated that the "bebeerine content" was about 89% and that the material comprised the total alkaloids of Pareira brava.

The free alkaloids were extracted with chloroform and the chloroform extract was recrystallized from chloroform - methanol to give d-isochondrodendrine (in a yield of 9%). Several months later the same procedure yielded only amorphous material, and more complicated fractional crystallization and chromatography on alumina was necessary to obtain additional isochondrodendrine; $[\alpha]_D^{21}$ +49° (c 0.59 in pyridine), $[\alpha]_D^{21}$ +118° (c 0.33 in N/10 hydrochloric acid); for two samples, m.p. 309° and 314°. The melting point of d-isochondrodendrine is variously reported from 290° to 316°.[20,51-54]

Anal. Calcd. for $C_{36}H_{38}O_6N_2$: C, 72.70; H, 6.44; N, 4.71. Found: C, 72.52; H, 6.50; N, 4.83.

1b. Isochondrodendrine Dimethiodide. — Isochondrodendrine in methanol and methylene chloride was refluxed with excess methyl iodide for one hour. The evaporated reaction mixture was crystallized from water to give double pyramids of the methiodide; $[\alpha]_D^{23}$ +60°, $[\alpha]_{5461}^{23}$ +84° (c 0.35 in water); m.p. 277°. The melting point is variously reported in the range of 275° to 287°.[20,51-54]

1c. O, O-Dimethyl Isochondrodendrine Dimethiodide. — Isochondrodendrine treated with methyl iodide in the presence of methanolic potassium hydroxide solution gave the dimethylether dimethiodide. Recrystallization from water gave colorless needles; m.p. 300°. Reported values include m.p. 302°,[53] and m.p. 294°.[52] The ultraviolet spectrum is reproduced in Figure 1.

Anal. Calcd. for $C_{40}H_{48}O_6N_2I_2$: C, 52.99; H, 5.34. Found: C, 53.04; H, 5.24.

1d. Cycleanine. — Isochondrodendrine in benzene and methanol was treated with ethereal diazomethane for two days. The product, chromatographed on alumina and eluted with benzene, was recrystallized from acetone to give needles of cycleanine; m.m.p. 274-276°. Reported values include m.p. 273-275°.[33]

1e. Reductive Cleavage of Cycleanine. — A 97-mg. portion of cycleanine was treated with sodium in liquid ammonia and the products were extracted with ether and sodium hydroxide according to the procedure described in Section III. K. 6f, to give 76 mg. of phenolic product and 10 mg. of nonphenolic product. The phenolic product was treated according to the method of Kidd and Walker to give armepavine oxalate; m.m.p. 211-213°. Conversion to the free base gave armepavine; m.m.p. 144°. The reported values include m.p. 211-212° and m.p. 145-146°, respectively.[33]

2. Tubocurarine

2a. Tubocurarine Iodide. — Commercial d-tubocurarine chloride (E. R. Squibb & Sons, U.S.P.) was converted to the iodide by treatment of an aqueous solution with potassium iodide. Recrystallization of the product from methanol - chloroform gave colorless crystals.

2b. O, O-Dimethyltubocurarine Iodide. — Treatment of tubocurarine chloride in methanol with methyl iodide and methanolic potassium hydroxide gave a product which after recrystallization from water had $[\alpha]_D$ +159° (c 0.27 in water); m.p. 263° dec. Reported values include $[\alpha]_D$ +160° and m.p. 267° dec.[53,55] The ultraviolet spectrum is reproduced in Figure 1.

3. Oxyacanthine

3a. Oxyacanthine Hydrochloride. — Commercial "oxyacanthine hydrochloride tetrahydrate" (Fluka AG., Buchs, Switzerland) gave a yellow aqueous solution. Addition of saturated sodium sulphate solution gave platelets of oxyacanthine sulphate. The latter was recrystallized from water and was converted to the hydrochloride by the addition of hydrochloric acid to an aqueous solution. The resultant oxyacanthine hydrochloride was recrystallized to constant specific rotation; $[\alpha]_D^{25}$ +188° (c 0.99 in water); K = 2.36 (in 0.5 M acetate buffer, pH 4.17 - chloroform). The infrared spectrum is reproduced in Appendix B.

3b. Oxyacanthine. — An aqueous solution of oxyacanthine hydrochloride was treated with ammonia and the resultant precipitate was collected by centrifuging, washed, and dried. Recrystallization from ether gave needles having m.p. 215-216°. Reported values are m.p. 217°,[56] m.p. 216-217°,[57] and m.p. 214-216°.[58]

3c. O-Methyloxyacanthine Hydrochloride. — One gram of oxyacanthine in 50 ml. of methanol was treated with 40 ml. of 0.5 M ethereal diazomethane. The next day a similar addition of diazomethane was made and after a total reaction time of two days at 5° the reaction mixture was evaporated. The products were dissolved in dilute hydrochloric acid and the solution was extracted with ether. The residual

ether was evaporated and hydrochloric acid was added to give 80 ml. of 5% hydrochloric acid solution, which gave 581 mg. (a 50% yield) of crystalline product. Recrystallization gave O-methyloxyacantine hydrochloride; $[\alpha]_D^{26}$ +226° ($\underline{c}$ 0.96 in water); $\underline{K}$ = 0.23 (in 0.5 $\underline{M}$ acetate buffer, pH 4.17 - chloroform).

4. Berbamine

4a. Berbamine. — Two grams of commercial "berbamine hydrochloride monohydrate" (Fluka AG.) dissolved in 50 ml. of 0.5% hydrochloric acid gave an amber solution. Addition of sodium nitrate solution gave an amorphous precipitate. A solution of the amorphous nitrate was treated with ammonia and was extracted with ether to give a colorless ether extract weighing 0.99 g. after evaporation. Crystallization from 35 ml. of benzene gave 0.91 g. of long needles of the benzene adduct of berbamine; m.m.p. 125-127°. Reported values are m.p. 127°,[56] and m.p. 124-126°.[59]

A 100-mg. sample of berbamine was dissolved in very dilute hydrochloric acid and the solution was warmed and blown with nitrogen to remove benzene. From the solution, which was further acidified to give approximately 10 ml. of 3% hydrochloric acid, were obtained 86 mg. of long, fine rods of berbamine hydrochloride. The infrared spectrum is reproduced in Appendix B.

4b. Isotetrandine. — Two grams of berbamine in 50 ml. of methanol were treated with a total of 115 ml. of 0.5 $\underline{M}$ ethereal diazomethane at 5° over a period of two days. The product could not be crystallized as the hydrochloride, hydrobromide, nitrate, sulphate, or oxalate. Crystallization of the free alkaloid from ether was not successful, and gradient elution chromatography employing benzene containing increasing amounts of methanol gave one main peak, portions of which gave no crystals from methanol, ethanol, or acetone.

A 2.14-g. portion of berbamine in 50 ml. of methanol at 5° was treated with a total of 130 ml. of ethereal diazomethane over a period of two days. The excess diazomethane was removed and the solution was evaporated to dryness. A benzene solution of the residue was extracted with 5% sodium hydroxide solution and with very dilute hydrochloric acid solution. The acid solution of the product was treated with ammonia and was extracted with benzene - ether (2:1). The benzene - ether solutions on evaporation to dryness gave 1.54 g. of amorphous product. Crystallization of the latter from acetone was not successful. Crystallization from ether was likewise not successful; but after the solution was evaporated to a syrup, a few crystals formed; and on the addition of a few drops of ether and stirring, additional crystalline material was obtained. Seeding of concentrated ethereal solutions with these crystals gave no additional crystals; however, evaporation of the mother liquor to a syrup and stirring in a few drops of ether yielded additional crystals, to give a total of 0.72 g. of crystalline product.

4c. Reductive Cleavage of Isotetrandine. — The 900 mg. of amorphous isotetrandine obtained in the original preparation was dissolved in 8 ml. of benzene -toluene (1:1) and was added dropwise over a period of 25 minutes to 150 ml. of liquid ammonia at $-40°$. The solution was kept blue by the addition of a total of 350 mg. of sodium and this blue color persisted for one-half hour after the addition. After the ammonia was allowed to evaporate overnight, the products were worked up as described in Section III. K.6f. The resultant ethereal solutions were dried over anhydrous magnesium sulphate and were evaporated to give 326 mg. of nonphenolic product and 365 mg. of phenolic product. Additional quantities of 33 mg. and 26 mg., respectively, were recovered from the drying agents.

The nonphenolic product was chromatographed on a 1.8 x 5-cm. column of 14 g. of Woehlm alumina of activity II. The first 100-ml. portion of benzene eluted almost all the material, and this eluate on evaporation to dryness and crystallization from 6 ml. of hexane gave 98 mg. of crystals, presumably l-O-methylarmepavine, which had been obtained by other investigators.[45]

The phenolic product was recrystallized twice from 20-ml. portions of benzene to give 224 mg. of light amber crystals, presumably d-N-methylcoclaurine.

5. Epistephanine

5a. Hydroepistephanine-A Hydrochloride. — A sample of epistephanine was supplied by Dr. Yasuo Watanabe, First College of Pharmacy, Fukuoka City. This material was recrystallized from methanol and was reduced by the method of Tomita and Watanabe.[60] A 504-mg. sample of epistephanine, dissolved in 7.5 ml. of 20% sulphuric acid and 7.5 ml. of ethanol, was treated with 900 mg. of zinc dust and refluxed on a water bath. At hour intervals, 1.5-ml. portions of 20% sulphuric acid and 150-mg. portions of zinc dust were added, and after five hours the warm mixture was filtered. Overnight cooling gave a crystalline precipitate, and additional crystals were obtained from the mother liquors on addition of ethanol, to give a total of 3.16 g. of crystalline material (A). The neutral mother liquor was evaporated at reduced pressure to a syrupy residue (B). Portions (A) and (B) were separately extracted in a system of chloroform and 5% aqueous sodium hydroxide. The chloroform layers were washed with water, dried with potassium carbonate, and evaporated at reduced pressure to give 225 mg. and 370 mg. of oily products from (A) and (B), respectively. Each portion was dissolved in 15 ml. of ethanol and five drops of concentrated hydrochloric acid were added to each solution. The solution from (B) gave very little crystals. The solution from (A) gave 163 mg. of crystals, which were recrystallized from 12 ml. of 0.5% hydrochloric acid to give 152 mg. of small, long rods of hydroepistephanine-A hydrochloride; $[\alpha]_D^{24}$ +300° (c 0.27 in water); $\underline{K}$ = 1.03 (in 0.5 $\underline{M}$ acetate buffer, pH 4.17 -chloroform); $\underline{R}_f$ = 0.58 (for the free alkaloids, see Section III.J.4a). The infrared spectrum is reproduced in Appendix B. Reported is $[\alpha]_D^{29}$ +298°.[60]

M. Toxicity and Resistance to Marine Borers

1. Pharmacological Studies

Pharmacological studies with rodiasine dimethiodide and with other quaternary derivatives of the greenheart alkaloids were carried out at the Medical College of Virginia under the direction of Professor Harvey B. Haag. The following investigations were made:

(a) Curare assays by the rabbit head-drop method as described in the U. S. Pharmacopeia

(b) Nerve-muscle studies with isolated frog muscles

(c) Lethal-dose determinations by intravenous injections in mice

(d) Blood-pressure and respiratory studies in anesthetized dogs

The results of studies (a), (b), and (c) are summarized in Table VI. The comparative index is a comparative safety factor which takes into consideration the comparative curarizing dose and the comparative lethal dose, but does not take into consideration possible side effects or possible differences in effect for different species of animals.

Studies on anesthetized dogs showed that for rodiasine dimethiodide and for rodiasine (W) methochloride 20% of the lethal dose was necessary to produce a depressive effect on the respiration, whereas for tubocurarine chloride 10% of the lethal dose produced a depression in respiration. Similarly it was found that, whereas tubocurarine chloride produces a depression in blood pressure at 40% of the lethal dose, rodiasine dimethiodide and rodiasine (W) methochloride do not give a direct depression in blood pressure at this concentration.

Some of the results of the pharmacological studies have been presented at the September 1954 meeting of the Pharmacological Society. The abstract of the paper[61] states:

> The crystalline dimethiodide of a new alkaloid from greenheart has been found to possess curare-like activity. By the rabbit head-drop method this compound possessed approximately one-half and by the frog nerve-muscle assay one-fourth the activity of d-tubocurarine, but only one-tenth the toxicity. A mixture containing the aforementioned and other quaternized material showed approximately one-eighth the activity of the reference standard in both the rabbit

head-drop and isolated frog nerve muscle preparation. Intravenous administration of the crystalline methiodide in near lethal doses caused little or no effect on blood pressures of pentabarbitalized dogs. Cruder preparations, on the other hand, caused marked hypotension. The ganglionic blocking activity of these compounds qualitatively resembled that of curare. Respiratory failure was the first immediate toxic effect of high doses of both crude and crystalline preparations. In contrast, mixed unquaternized alkaloids showed no curare-like activity in rabbits in doses up to 16 mg./kg. The LD_{50} of the crystalline dimethochloride compound was 1.08 mg./kg. in contrast to 0.12 mg./kg. for d-tubocurarine chloride administered to mice intravenously under the same conditions.

2. Toxicity Tests

The toxicities of the greenheart alkaloids to adult Limnoria and to Teredo larvae were determined. The test animals were placed in very dilute sea-water solutions of the alkaloids and the concentrations of alkaloids necessary to kill a certain percentage of the animals in a specified time were determined. A detailed report has been made of the procedures which were employed.[62]

The toxicity of the alkaloids to Limnoria was found to be comparatively low. At concentrations of 100 p.p.m. (parts per million) the alkaloids had no effect on Limnoria. For several other alkaloids and for a number of plant extractives, including a chloroform extract of Ocotea rubra[63] and alcoholic, acetone, aqueous, and ethereal extracts of greenheart, similar low toxicities were observed.

The greenheart alkaloids were considerably more toxic to Teredo larvae. In an earlier series of tests, purified dirosine, isoocoteamine, and demararine (the most abundant of the ether-soluble alkaloids) were toxic at concentrations of 10 p.p.m., whereas other mixtures, plant extracts, or alkaloids required concentrations of 100 p.p.m. or greater to produce 100% kill in three days. Under similar conditions, a concentration of 25 p.p.m. of creosote was required to produce the same results.

The results of later series of tests are listed in Table VII. Under the conditions of Test Series A, the minimum lethal concentration for creosote, like that of the ether-soluble alkaloids, was less than 1.6 p.p.m. In comparative tests, the toxicity of creosote and of the ether-soluble greenheart alkaloids were thus again of the same order of magnitude.

Table VI. Pharmacological Tests

Sample	Curare Activity, % of Reference Activity[a]	Frog Nerve-Muscle Test, % of Reference Activity[b]	Lethal Dose, % of Reference Activity[c]	Comparative Index[d]
d-Tubocurarine Chloride	100.0	100.0	100.0	1.0
Rodiasine Dimethiodide	37.3	17.7	9.8	3.8
Rodiasine Dimethochloride[e]	47.0	22.2	12.3	3.8
Rodiasine (W) Methochloride[f]	37.3	16.8	11.1	3.4
Quaternary Methiodides (G)[g]	13.4	9.1	3.9	3.5
Quaternary Methiodides (W)[g]	13.4	7.6	5.0	2.8
Methiodides of Crude Bases	13.7	9.1	3.8	3.7
Hydrochlorides of Alkaloids (G)	<1.5	6.4	0.3	—

a/ Curarizing dose of d-tubocurarine chloride = 0.179 mg./kg.

b/ Comparisons made directly with tubocurarine chloride.

c/ LD$_{50}$ of d-tubocurarine chloride = 0.12 mg./kg.

d/ Comparative index = Comparative curare activity ÷ Comparative lethal dose.

e/ Calculated from values for the methiodide.

f/ A mixture of rodiasine and isorodiasine dimethochlorides.

g/ Methanol-soluble methiodides from ether-soluble alkaloids (G) and (W).

Table VII. Toxicities to *Teredo* Larvae

Agent	MLC, [a] p.p.m.	MLC, [b] p.p.m.	MLC, [c] p.p.m.
From Greenheart Bark			
Hydrochloride of:			
Crude Bases	6.3		
First Ether Extract	<1.6		
Second Ether Extract	<1.6		
Ether-Insoluble Crude Bases	>12.5		
Rodiasine		0.25	0.125
Norrodiasine		0.25	0.5
Dirosine	<1.6	0.25	0.5
Isoocoteamine	3.2	0.25	>1.0
Demerarine	3.2		
From Greenheart Sawdust			
Hydrochloride of:			
Crude Bases	6.3		
First Ether Extract	<1.6		
Ether-Insoluble Residue	6.3		
Other Alkaloids			
Hydrochloride of:			
Oxyacanthine		1.0	>1.0
Berbamine		1.0	>1.0
Isochondrodendrine	6.3		
Strychnine	6.3		
Berberine	6.3		
Other Agent			
Creosote	<1.6		

[a] Test Series A. Minimum Lethal Concentration, in parts per million, for 100% kill in five-day exposure.

[b] Test Series B. Minimum Lethal Concentration, in parts per million, for 100% kill in four-day exposure.

[c] Test Series C. Minimum Lethal Concentration, in parts per million, for 90% kill in four-day exposure.

3. Harbor Tests

Small panels of greenheart and of other woods were exposed to marine boring organisms in sea water as part of a series of harbor screening tests of marine borer inhibitors. The 1/4 x 1-7/8 x 6-inch panels listed in Table VIII were exposed in Port Hueneme harbor starting in May 1957.[64] Some panels were exposed at subsequent dates and were thus subjected to shorter exposure. Panels which were damaged to a point at which their loss appeared imminent were removed from test.

Greenheart panels extracted with a number of solvents are also listed. These extractions were carried out at the boiling points of the various solvents.

Other samples listed in Table VIII include a few highly resistant tropical woods and some domestic woods. The lignum vitae samples had been cut across the grain, whereas all other samples listed were cut parallel to the grain.

Also included in the table are southern yellow pine panels impregnated with creosote to the indicated retentions and similar panels impregnated with a cellosolve solution containing 2% of the ether-soluble alkaloids from greenheart wood to give a 2% alkaloid content in the panels.

Some samples were also exposed at an NCEL test site at Pearl Harbor.[64] Four greenheart panels exposed at Pearl Harbor were removed from test after 7, 14, 15, and 15 months, respectively, because of heavy Martesia attack, but during this time they suffered no attack from Limnoria or Teredo. Of eight creosoted panels, seven panels were removed after 17, 20, 29, 48, 55, 59, and 59 months, respectively, because of moderate to very heavy attack by Limnoria but with only very light Martesia and no Teredo attack; one panel had not failed after 59 months.

Table VIII. Harbor Test Results

Wood	Exposure Time, mo.	Damage by[a]	
		Limnoria	Teredo
Greenheart	60	0	4
Greenheart	43	0	4
Greenheart	39	0	3
Greenheart Extracted With:			
Acetic Acid	60[b]	0	3
Chloroform	51[b]	1	4
Ether	56[b]	1	5
Methanol	51[b]	0	4
Sea Water	14[b]	4	6
Other Tropical Woods			
Afambeau	43	0	0
Antidesma Pulvinatum	33	0	0
Lignum Vitae	60	0	3
Domestic Woods			
Douglas Fir	4[b]	6	2
Ponderosa Pine	3[b]	3	5
Southern Yellow Pine	4[b]	5	4
Southern Yellow Pine Impregnated With:			
Greenheart Alkaloids (2%)	10	3	0
Creosote (36 lb./cu. ft.)	70	4	0
Creosote (40 lb./cu. ft.)	63	5	0
Creosote (33 lb./cu. ft.)	61	2	1
Creosote (37 lb./cu. ft.)	56	5	0
Creosote (30 lb./cu. ft.)	53	5	0

a/ Damage at Port Hueneme by Limnoria and Teredo, rated visually as follows:
0 = no attack; 1 = trace (sometimes indefinite); 2 = very light; 3 = light;
4 = moderate; 5 = heavy; 6 = very heavy. Average values are listed for
groups of two to six panels exposed at Port Hueneme.

b/ Removed from test after panels failed.

IV. DISCUSSION

A. Bebeerine

According to authoritative reviews of alkaloid chemistry[12, 13] and according to general scientific belief, greenheart contains the alkaloid "bebeerine, " or chondrodendrine, whose structure has been well established. However, all pure crystalline "bebeerine, " including all that employed for structure determinations, has been obtained from Pareira brava or from Chondrodendron species.

Maclagan[8, 65] and others[9, 66] had obtained bebeerine from greenheart by extracting the ground bark with acid, precipitating the crude bases with alkali, and extracting the dried crude bases with ether. The partially purified ether extract was called bebeerine and the residue was called sipeerine. Later, Boehm[16] and others[15, 18, 21, 55] in a similar manner obtained the ether-soluble alkaloids from Pareira brava, tubo-curare, or Chondrodendron species, and by crystallization of these ether-soluble alkaloids from methanol, they obtained crystalline "bebeerine." The belief that this crystalline alkaloid is the same as that which occurs in greenheart appears to be founded entirely on a comparison made by Flückiger in 1869.[11]

Flückiger had found that the bebeerine from Nectandra rodiaei had the same properties as pelosine from Cissampelos pareira. He further believed it very probable that these alkaloids were also the same as paricine, chinoidine, and buxine, and that they should all be called by the latter name. The properties which these five amorphous alkaloids had in common, however, were properties which are shared by quite a number of alkaloids. (It is of interest that a 20% solution of Flückiger's pelosine in acetone gave a specific rotation of +30°, whereas "bebeerine" is reported to have a specific rotation of +298° in methanol and β-bebeerine is reported to have a specific rotation of +29° in ethanol.)[13] Scholtz[21] referred to Flückiger's comparison of bebeerine and pelosine and reported that "bebeerine" could be obtained in crystalline form from Bebeerinum purum, a commercial powder. (This crystalline material had a melting point of 214° and a 1.6% solution in absolute alcohol had a specific rotation of -298°.

Faltis and Neumann[14] challenged the assertion of Flückiger and others who claimed that bebeerine from greenheart is the same as the alkaloid obtained from Pareira brava. They considered that the comparison between pelosine and bebeerine

was not valid and noted that Boedecker[66] had believed these materials to be different on the basis of olfactory tests on pyrolysis products. Furthermore, they stated that Cissampelos pareira was the false pareira. On the basis of the early literature, they further concluded that bebeerine from greenheart was a nonphenolic base whereas the crystalline alkaloid from Pareira brava contained phenolic groups. Faltis and Neumann pointed out that the Bebeerinum purum, which was used as a drug against undulating fever and as a quinine substitute, was originally obtained from green-heart bark, but toward the end of the nineteenth century Pareira brava was increasingly substituted as a source of the drug. They suggested that for etymological reasons the term bebeerine be used only for the alkaloid obtained from greenheart, and that the pure alkaloid from Pareira brava and Chondrodendron species be called chondrodendrine.

Unfortunately the suggestion of Faltis and Neumann has met little acceptance, and the term "bebeerine" is commonly used for the alkaloid chondrodendrine.[12,53,54,67] A wide variety of other usage complicates the matter. Bebeerine is defined as an amorphous alkaloid obtained from the bark of the bebeeru (Nectandra rodioei).[68] Commercial bebeerine is described as a mixture of alkaloids which includes α-bebeerine, β-bebeerine, etc.[68] Merck Index[69] defines "bebeerine hydrochloride" as the total alkaloids from the root of Chondrodendron species. Henry[13] states that bebeerine has been described under various names: pelosine, chondrodendrine, chondodendrine, and curine (for the l-form).

In order to study the effects of the alkaloid on marine wood-boring organisms, it was desired to obtain chondrodendrine from Pareira brava or other sources, and, if possible, to obtain the same alkaloid from greenheart. Pareira brava roots were extracted, as described in Section III. A, but no chondrodendrine could be isolated from the ether-soluble alkaloids which they contained. A source of commercial "Bebeerine Hydrochloride" was located in Scotland, but this material yielded no crystalline "bebeerine." It was found that this commercial material consisted merely of the crude alkaloid hydrochlorides from Pareira brava. Pareira brava is reported to give crystalline "bebeerine" in some instances and not in others.[15] Although "bebeerine," or chondrodendrine (formula I), was not obtained, the second source did yield the alkaloid isochondrodendrine (II).[70]

Attempts to obtain crystalline bebeerine from greenheart were also unsuccessful. The ether-soluble alkaloids were isolated from two samples of greenheart bark obtained from different sources. No crystals could be obtained from a methanolic solution of these alkaloids. Attempts to obtain crystals from a variety of other organic solvents were likewise fruitless.[70]
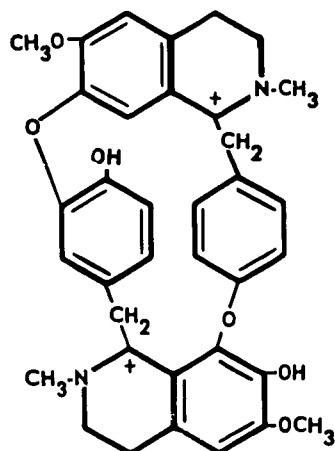
I
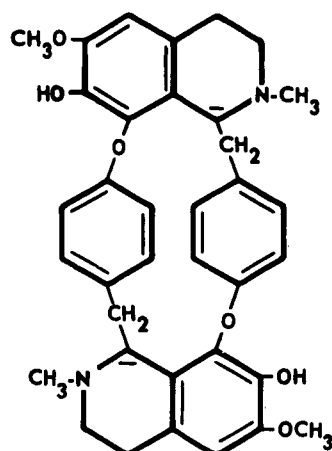
II

Von Planta[9] precipitated the ether–soluble alkaloids from greenheart with basic lead acetate and reextracted the dried product with anhydrous ether. He dropped a concentrated alcoholic solution of this product into water and dried the resulting white precipitate to obtain reportedly pure bebeerine.

The amorphous ether–soluble alkaloids isolated in the present study had the superficial characteristics of bebeerine as described by von Planta. However, the analyses did not agree with von Planta's formula of $C_{18}H_{21}O_3N$, and the broad melting range of 180–215° was higher than the reported value of 180°.

The amorphous product furthermore did not have the properties expected of pure crystalline "bebeerine," or chondrodendrine. Although the carbon, hydrogen, and nitrogen values agreed with those calculated for the formula $C_{36}H_{38}O_6N_2$, the methoxyl content was almost twice that expected for the two methoxyl groups of chondrodendrine. Furthermore, the specific rotation of +129° was much lower than the reported value of +298°.[16]

Thus the product appeared to be identical neither with the pure compound "bebeerine," or chondrodendrine, nor with the reportedly pure bebeerine obtained by von Planta. The fact that fractional crystallization from a variety of organic solvents gave a number of fractions having different physical properties, indicated that the amorphous product was a mixture which might be quite complex. Various amounts of all the five greenheart alkaloids reported by Maclagan[8, 10, 65] might be included and it is quite possible that none of these were pure compounds but that each was a mixture.

The laboratory experiments described above as well as a thorough search of the literature gave no evidence that chondrodendrine is present in greenheart. In fact, there appeared to be no substantial evidence that pure alkaloids of any type had in the past been isolated from greenheart.

Thus, the name "bebeerine" was originally employed for a mixture of alkaloids from greenheart, later for other mixtures of alkaloids, and still later for a pure alkaloid which apparently does not exist in greenheart. Although the use of this name for its original application might be justified on etymological grounds, the complete abandonment of the name "bebeerine" appears desirable.

B. The First Pure Alkaloid From Greenheart

As indicated above, very little was actually known about the alkaloids of greenheart. Any protective action of the greenheart alkaloids could therefore not be ascribed to a well-characterized alkaloid ("bebeerine"), and a further investigation of the alkaloids was undertaken.

The present study of the alkaloids of greenheart was limited primarily to ether-soluble alkaloids from greenheart bark, rather than from the wood. The reasons were as follows: (a) previous work had been carried out with the ether-soluble alkaloids from the bark; (b) the bark has a higher alkaloid content than the wood. It is expected that the types of alkaloids present in the wood and in the bark would be quite similar even though the specific amounts of individual alkaloids may vary somewhat. This expectation appeared to be borne out by toxicity studies with Teredo larvae, which showed equal toxicities for the ether-soluble alkaloids from either source. The alkaloids less soluble in ether were found to be less toxic.

The ether-soluble alkaloids from greenheart bark gave negative tests for sulphur, halogen, and phosphorus and also for a variety of functional groups including aldehydes, esters, methylenedioxy groups, enols, and phenols with free para positions. Furthermore, there appeared to be no groups which were readily hydrolyzed by acid or alkali. Positive tests for phenols were obtained with Folin's reagent and with Millon's reagent. The ultraviolet spectrum, Figure 1, shows the presence of a single predominant peak at 283 mμ, typical of phenols and phenyl ethers. This data is not inconsistent with the properties of the general class of bisbenzylisoquinoline or biscoclaurine alkaloids of which chondrodendrine is a member.

Attempts to obtain crystalline alkaloids by fractional crystallization were hampered by the instability of the alkaloids. They were readily oxidized in solution and were decomposed by light, and it appeared that considerable decomposition products were formed in the crystallization experiments. .

The first crystalline alkaloid obtained from greenheart was a methiodide derivative. Treatment of the ether-soluble alkaloids with methyl iodide gave a mixture which was not completely soluble in methanol. The precipitate which was formed was crystalline and on recrystallization from methanol it melted at 321° with decomposition. The elementary analyses for this methiodide appeared consistent with an empirical formula $C_{40}H_{50}O_6N_2$ for the positive ion. This material was designated rodiasine dimethiodide.[71]

The data suggested the possibility that the quaternary rodiasine dimethiodide was derived from a di-tertiary alkaloid, originally present in the extraction liquor. The latter alkaloid, with two less methyl groups, would have the empirical formula $C_{38}H_{44}O_6N_2$. Because of the uncertainty in the number of hydrogens, the formula could also be $C_{38}H_{42}O_6N_2$, which is the same as the empirical formula of the dimethyl ether of chondrodendrine or a structural isomer. This possibility, however, was tentatively eliminated on the basis of the positive phenol tests. The existence of a free phenolic group was confirmed by the preparation of the O-methyl derivative of rodiasine dimethiodide.

The absorption spectra of rodiasine dimethiodide and its O-methyl derivative were compared with those of other biscoclaurine alkaloids. The spectra of O-dimethyl-d-tubocurarine iodide and O-dimethyl-d-isochondrodendrine dimethiodide, Figure 1, as well as those of d-tubocurarine iodide and d-chondrocurine iodide[72] all showed well-defined maxima at 225 mμ. These alkaloids or alkaloid derivatives are all members of the chondrodendrine or isochondrodendrine series of biscoclaurine alkaloids. Whether maxima in the 225-mμ region are typical of this series of alkaloids and whether other series of biscoclaurine alkaloids also show similar maxima had not been established. (See further Section IV. D, page 87.) Most of the reported absorption spectra for biscoclaurine alkaloids show only the portion of the spectra above 240 mμ.

Rodiasine dimethiodide did not show a maximum at 225 mμ. In the region above 240 mμ the absorption curve was not inconsistent with those reported for a number of biscoclaurine alkaloids.[35, 73-75]

There was not at this point sufficient evidence to place rodiasine dimethiodide in the biscoclaurine or bisbenzylisoquinoline series of alkaloids. A minimum C_{40}, rather than a C_{20}, formula was indicated by the presence of one phenolic group for every 40 carbon atoms as shown by the formation of an O-methyl derivative and by methoxyl determinations which showed the presence of four methoxyl groups in rodiasine dimethiodide. The absence of alkoxyl groups, other than methoxyl, was shown by the infrared spectrum of the alkyl iodide produced in the alkoxyl determination. Involvement of the one remaining oxygen in an ether linkage was suggested by

negative tests for ester, amide, and carbonyl groups. This ether linkage could be the diphenyl ether linkage of a biscoclaurine alkaloid. If rodiasine dimethiodide is a biscoclaurine alkaloid, the empirical formula of the di-tertiary precursor, rodiasine, is $C_{38}H_{44}O_6N_2$, and the most probable formulas for O-methylrodiasine are III, IV, V, and VI. Each of these structures has two optically active centers, indicated by an asterisk, and can thus exist in four configurations.
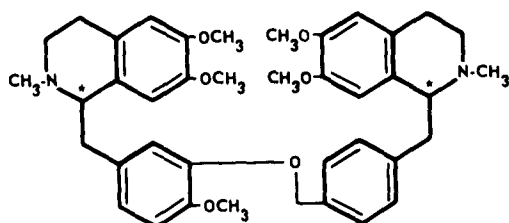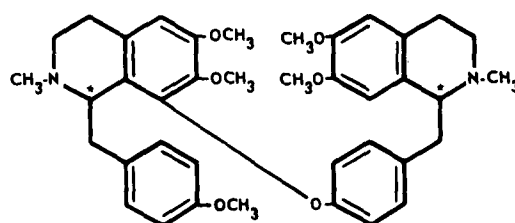

III
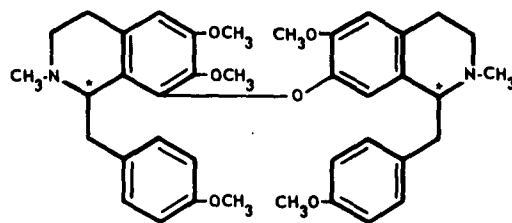
IV
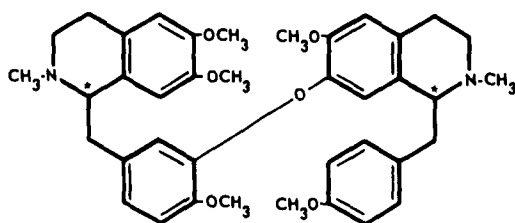
V

VI

From the ether-soluble alkaloids (W), an insoluble methiodide was obtained which melted at 314° with decomposition. It was designated rodiasine (W) methiodide. Upon O-methylation, this material gave O-methylrodiasine dimethiodide. It thus appeared that the lower-melting methiodide was a compound which had one methoxyl group and one phenolic group interchanged with the corresponding groups of rodiasine dimethiodide, or alternatively, that the lower-melting methiodide was a mixture of such a compound and of rodiasine dimethiodide.

Conversion of rodiasine (W) methiodide to the methochloride and subsequent fractional crystallization gave two different methochlorides, melting at 309° and at 283° with decomposition. The latter of these was converted back to a methiodide which melted at 320° with decomposition, and which, therefore, was rodiasine dimethiodide. The lower-melting methiodide therefore is a mixture of rodiasine dimethiodide and another isomeric methiodide which was designated isorodiasine dimethiodide.

The methiodides of biscoclaurine alkaloids have curare-like activity.[76,77] In a collaborative study with Professors Harvey Hague, Paul F. Larsen, and J. K. Finnigan of the Medical College of Virginia, it was found that the methiodides of the crude ether-soluble alkaloids had marked curare-like activity. The activity of rodiasine dimethochloride was considerably greater than that of the above methiodides and was approximately one-half that of a standard U.S.P. reference sample of tubocurarine chloride. The toxicity of the rodiasine dimethochloride was much less than that of tubocurarine chloride, and the comparative index, or safety factor, was therefore almost four times that of tubocurarine chloride, as shown in Table VI. It is of interest that tubocurarine chloride is used extensively in certain types of surgery as a muscle relaxant and that on the basis of the above studies a compound like rodiasine dimethochloride might be more desirable pharmacologically.

C. Isolation of Other Alkaloids

Rodiasine dimethiodide had been obtained from the methiodides of the ether-soluble alkaloids of greenheart bark and the presence of isorodiasine dimethiodide had been shown. However, no other pure methiodide could be obtained by fractional crystallization from methanol or water. Additional methods of separating the methiodides were not investigated. Isolation of the alkaloids as their methiodide derivatives had the disadvantage that the methiodides cannot be reconverted to the alkaloids originally present in greenheart.

Although it was not possible to crystallize the alkaloids directly from organic solvents, it was found that in some instances crystalline hydrochlorides could be obtained from aqueous dilute hydrochloric acid solutions of the ether-soluble alkaloids. Such crystallizations of relatively pure alkaloid hydrochlorides from the mixture of hydrochlorides were, however, very unpredictable and, furthermore, the crystallizations were extremely slow. In some instances the ether-soluble alkaloids obtained from the first acid extracts of the bark were not completely soluble in 5% hydrochloric acid. The insoluble residues readily gave a crystalline hydrochloride on recrystallization. In other instances hydrochloric acid solutions of the ether-soluble alkaloids, which had remained in the refrigerator for several years, slowly deposited crystals. Recrystallization of either the former or of the latter crystals gave a pure alkaloid hydrochloride which had a specific rotation of +250°. It was tentatively called alkaloid B hydrochloride.

Some samples of crude alkaloid B hydrochloride appeared to be only partially soluble in warm water. The residue was shown to be an amphoteric material which was only slightly soluble in dilute hydrochloric acid or in alkali. Recrystallization from very dilute hydrochloric acid, to a constant specific rotation of +72°, gave a new alkaloid tentatively designated as alkaloid C hydrochloride.

The very slight solubility of alkaloid C hydrochloride in aqueous alkali suggested a kryptophenolic character and the possibility that the ether-soluble alkaloids might contain other kryptophenolic alkaloids. When a chloroform solution of the ether-soluble alkaloids was extracted with aqueous alkali, the phenolic alkaloids recovered were only one-third of the starting material. Two-thirds of the alkaloids were thus nonphenolic or kryptophenolic.

Observations in the early literature [8,9] indicated that the ether-soluble alkaloids of greenheart were insoluble in alkali, and these reports led Faltis and Neumann[14] to believe that "bebeerine" from greenheart is a nonphenolic material. However, in the present studies the ether-soluble alkaloids had given positive phenol tests, and an acid solution of these alkaloids gave very little precipitate on the rapid addition of alkali. It is also of interest that an aqueous solution of the alkaloid hydrochlorides did not give an immediate precipitate upon the addition of a small quantity of silver nitrate. The formation of a complex with the silver ion was thus indicated.

The direct crystallization of alkaloid hydrochlorides from the ether-soluble alkaloid mixture was too slow and too uncertain to be useful as a practical method for the isolation of the various alkaloids. The hydrochlorides of alkaloids B and C were obtained only in some instances. One crystallization yielded a mixture which appeared to consist predominantly of alkaloid D hydrochloride and another crystallization yielded a product apparently consisting primarily of alkaloid E hydrochloride.

The countercurrent distribution curve of the ether-soluble alkaloids, obtained with 9 mg. of alkaloid hydrochlorides and with a 54-tube stainless-steel Craig apparatus,[30] had three main peaks and one secondary peak, as shown in Figure 2. It appeared from the curve that a larger-scale separation employing a smaller number of tubes would not give very good separation. However, it was considered possible that there would be sufficient purification or concentration of some of the alkaloids so that crystallization from the various fractions might be possible.

The larger-scale, ten-transfer distribution of 10 g. of alkaloid hydrochlorides gave the curve shown in Figure 3. From fractions 3 to 5, a new crystalline alkaloid hydrochloride was obtained in a 1% yield, based on the total starting material. This hydrochloride had a specific rotation almost identical to that of alkaloid C hydrochloride (+73° vs. +74°), but its distribution coefficient was considerably lower (0.7 vs. 2.0). This new hydrochloride was tentatively designated alkaloid D hydrochloride.

From fractions 6 to 8 of the above distribution a further new crystalline hydrochloride was obtained. It had a specific rotation (+98°) different from that of the other hydrochlorides and it was tentatively designated alkaloid E hydrochloride.

Fraction 10 of the above distribution was subjected to a new ten-transfer distribution in a more alkaline system. The curve obtained is shown in Figure 3. From the new fractions 2 to 6, crystals were obtained which appeared to consist of two new alkaloid hydrochlorides. The amounts obtained were too small to warrant further investigation.

Preparative scale countercurrent distribution studies were discontinued since better results were obtained in the chromatographic work described below.

In initial chromatography experiments a variety of solvents and aluminas of various activities were employed. Partial separations were obtained but only one peak was obtained with each new eluent. The chromatography curves were obtained from the absorbances of the eluates or from the weights of the fractions. The separations were evidenced by the shapes of the curves, and by the differences in the specific rotations of the fractions obtained. With small samples and comparatively large volumes of eluent, and with the aid of a fraction collector, only one peak again was obtained with each eluent employed, and the chromatograms had very long trailing edges.

The chromatographies on alumina appeared to be accompanied by some decomposition. This decomposition was indicated by a lowering of the average specific rotation of the products as compared to that of the original material. In some instances the decomposition was indicated by an increase in weight, giving a yield of over 100%, in other instances by the only partial solubility of the product in hydrochloric acid. In order to help reduce any possible decomposition caused by the alumina, neutral alumina was employed in all but the initial experiments. In order to help reduce the effects of solvents and of dissolved oxygen, preparative scale chromatographies were carried out with the minimum amount of solvent considered necessary for achieving a reasonable degree of separation, and as rapidly as appeared practical.

Ten grams of alkaloid were chromatographed in the conventional manner; that is, by the stepwise addition of a series of eluents of increasing eluting power. The eluents were methylene chloride, mixtures of methylene chloride containing increasing amounts of methanol, and finally methanol. As shown in Figure 4, only a single peak was obtained with each new eluent, but the shapes of the trailing edges indicated the presence of more components than peaks.

It appeared that much better separation than that obtained above should be possible by employing an eluent of gradually changing composition. Such an eluent would not only tend to eliminate sharp fronts and to reduce the tailing of peaks, but would also make it possible to predetermine the amount of solvent to be employed and would eliminate the problem of when to change solvents. The methanol content of this eluent should rise very slowly at first and quite rapidly at the end, in an exponential manner. A linear or convex change would be less desirable in this particular instance, because it would give too rapid an elution at the beginning or too slow an elution at the end of the chromatography.

Some of the theoretical advantages of gradient elution chromatography have been discussed by others [78-81] and a number of methods for producing gradient eluents have been described. [78, 79, 82, 83] None of the described methods gave an eluent of the desired exponential composition curve, nor were they well suited for producing eluents in quantities of 25 liters or more, as required for the large-scale separation.

The desired gradient eluent was produced with the aid of a constant-volume mixer[82] as described in Appendix A. With this new method, it is possible to produce an eluent having a composition curve which approximates, by a series of nearly straight lines, almost any desired shape.

Gradient eluent chromatographies of 30-g. portions of ether-soluble alkaloids gave continuous curves which indicated the presence of at least eight components. The curves obtained and the composition of the eluent employed are shown in Figure 5. The eluate from a typical run was divided into 17 fractions, as shown in Figure 5, and the alkaloids were isolated as the hydrochlorides. Three such chromatographies were run in as nearly identical manner as possible and the 17 corresponding fractions from each run were combined.

Crystalline hydrochlorides were obtained from a majority of the fractions, as shown in Table I. Fractional crystallization gave eight different alkaloid hydrochlorides which were purified to constant specific rotation. These were tentatively designated alkaloids C, D, E, F, G, H, I, and J hydrochlorides. [84] Their physical properties were listed in Table II. Alkaloid E hydrochloride was the most predominant alkaloid isolated and was obtained in a crude yield of 11% of the ether-soluble alkaloid mixture.

In following the course of the fractional crystallization of the alkaloid hydrochlorides, it was advantageous to determine their distribution coefficients as well as the specific rotations and decomposition points. The specific rotations of different alkaloids were in some cases quite similar. Some of the decomposition points were also quite similar, and they had the additional disadvantage of being somewhat dependent on the rate of heating and of not producing depressions on mixing.

The distribution coefficient ($\underline{K}$) was calculated from the absorbance of a very dilute solution of the alkaloid in aqueous acetate buffer before and after shaking with chloroform. The portion of the total sample remaining in aqueous layer is equal to the ratio of the final absorbance ($\underline{A}_f$) to the original absorbance ($\underline{A}_o$), provided the sample follows Beer's Law and is a pure material or a mixture of materials having the same extinction coefficients. Since $\underline{K}$ is equal to the ratio of the portions of the sample in the upper and in the lower layers, $\underline{K} = (\underline{A}_f/\underline{A}_o)$ ÷ $(1 - \underline{A}_f/\underline{A}_o)$, or $\underline{K} = \underline{A}_f/(\underline{A}_o - \underline{A}_f)$.

Distribution coefficients obtained for the various alkaloid hydrochlorides and hydrochloride mixtures at pH 4.17 ranged from about 0.15 to 14. Two batches of partially purified hydrochlorides, which had specific rotations of +75° and +74° and decomposition points of 290° and 285°, were shown to consist of different materials by their distribution coefficients of 0.83 and 2.5. Values obtained at pH 5.25 for the hydrochlorides of alkaloids B and G were 1.91 and 1.45, respectively, as compared to 10.5 and 9.1 at pH 4.17, and they helped to establish a difference between these two alkaloids.

In addition to the eight alkaloids which were isolated as crystalline hydrochlorides from fractions 4 to 14, other alkaloids which were not isolated must be present in the chromatography fractions. Fractions 1 and 2 are represented by different peaks on the chromatography curve and must, therefore, contain different alkaloids, which are not identical with the others that have been isolated. A small amount of crystalline hydrochloride was obtained from fraction 2 in the presence of chloroform. It had a specific rotation of +165° and a distribution coefficient of 0.15, but was not investigated further. The predominant alkaloids in fractions 1 and 2, which have very low distribution coefficients, are not identical with alkaloid B, because the hydrochloride of the latter has a very high distribution coefficient. If none of the above alkaloids is a decomposition or rearrangement product, the ether-soluble greenheart alkaloids therefore contain at least ten other alkaloids in addition to alkaloid B.

Alkaloid B was obtained by direct crystallization of the ether-soluble alkaloids from two sources of greenheart bark, but it was not isolated from the chromatography fractions, nor was it obtained by direct crystallization of the mixture of alkaloids which was chromatographed. The latter alkaloids were obtained from a different batch of bark. The alkaloid content of greenheart bark thus appears to vary considerably.

The specific rotation of chondrodendrine hydrochloride, calculated from data in the literature,[53,54] is approximately +253°. The melting point is reported as 265-266° dec.[53] and as 275° dec.[85] Although alkaloid B hydrochloride has a specific rotation of +250°, its decomposition point of 285° is higher than the above values.

None of the other eight alkaloid hydrochlorides isolated had the properties described for the dihydrochloride of chondrodendrine, and there still is no evidence that the latter alkaloid exists in greenheart.

D. Characterization of the Alkaloids

Alkaloid B hydrochloride and the eight crystalline hydrochlorides obtained from the chromatography were initially characterized by their specific rotations, by their decomposition points, and by their distribution coefficients. The method which was devised to determine the distribution coefficients gave results which were quite reproducible. The accuracy of this method depends chiefly on the accuracy of the spectrophotometric measurements. If the absorbances can be determined with maximum errors of 0.002 units, if the absorbances of the upper layers are read, and if there are no other significant errors, the expected limits of error of $\underline{K}$ would be 5% at $\underline{K}$ = 0.1, 3.6% at $\underline{K}$ = 0.33, 3% at $\underline{K}$ = 1, and 5% at $\underline{K}$ = 3. The measured values were reproducible within these limits.

The equivalent weights of the alkaloid hydrochlorides were determined by titrating aqueous solutions with sodium hydroxide. For initial determinations, 125-mg. quantities of the hydrochlorides were employed. Since some of the alkaloids were available in rather small quantities, a new procedure was developed, in which 50-mg. quantities of the hydrochlorides were titrated under an atmosphere of nitrogen. To obtain the equivalent weight of the free alkaloid, 36.5 was subtracted from the equivalent weight of the hydrochloride. This method, of course, did not take into consideration any possible hydration of the hydrochlorides.

The equivalent weights thus found for seven of the alkaloids, as listed below, suggested that these were members of a homologous series. The differences of approximately 7, rather than 14, between the groups of values indicated that the molecular weights should be twice the equivalent weights. If the alkaloids were all phenolic, the highest member of this series should have an equivalent weight of 320, a value which was later found for O-methylated alkaloid E. Such a series might be a series of biscoclaurine alkaloids with one diphenyl ether linkage, of which rodiasine might be a member. The values calculated for such a series are listed below, together with the values found for some of the greenheart alkaloids.

Alkaloid	Equivalent Weight	
	Found	Calculated
B, F, G	299, 297, 298	298
E, J	307, 307	305
H, I	313, 313	312
O-Methylated E (2)	320	319

The calculated values for a series of biscoclaurine alkaloids with two diphenyl ether linkages, such as that of which chondrodendrine is a member, would be 311 for the fully methylated alkaloid and 304 and 297 for lower members of the homologous series.

The most likely structures of a completely O- and N-methylated biscoclaurine alkaloid with one diphenyl ether linkage have been shown as formulas III to VI, page 74. The most probable formulas for completely O- and N-methylated bisco-claurine alkaloids with two diphenyl ether linkages are VII to X, below. Each of these eight formulas has two asymmetric carbon atoms, indicated by plus (+), by minus (-), or by an asterisk (*). Each of these asymmetric carbon atoms can have two possible configurations. Therefore each formula represents four possible isomers, and 32 structures are thus included in the most likely possibilities.
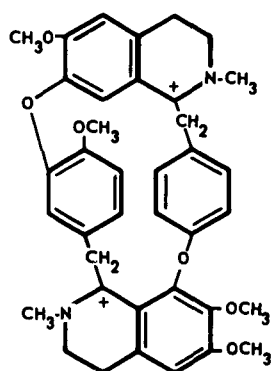
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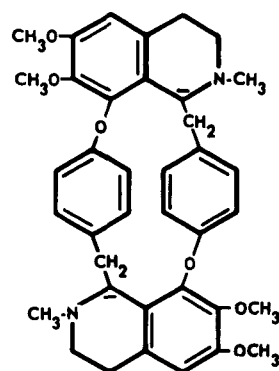
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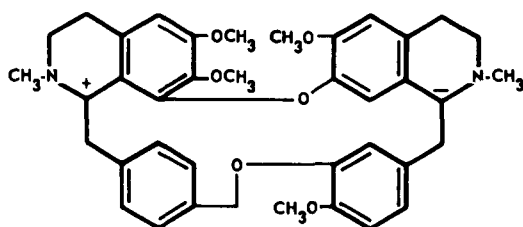
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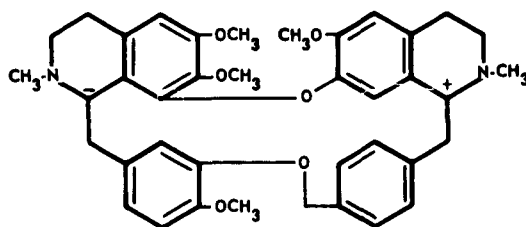
X

The paper partition chromatography of the greenheart alkaloids was investigated. Of a number of systems which were tried, the best was found to be amyl alcohol, pyridine, and water (110:110:90) on paper impregnated with potassium dihydrogen phosphate.[33] The chromatogram was developed by exposure of the paper to iodine vapor.

With the above system, the alkaloids could be divided into two groups. One group consisting of alkaloids B, F, G, and H had R_f values of 0.31 to 0.35. The other group consisting of alkaloids C, D, and E, had R_f values of 0.40 to 0.46. (The R_f is the ratio of the distance traveled by the spot to the distance traveled by the solvent front.) The differences in R_f values were not great enough to allow identification of the individual alkaloids within each group.

Alkaloid I gave two strong spots, which indicated the presence of two major components, one from each of the two different groups. Alkaloid J gave a strong spot and a weaker second spot, which indicated the presence of a component from the second group. Alkaloids B and C gave weak second spots, which could have been due to impurities formed by decomposition during the handling of the alkaloids. This method is thus capable of dividing the alkaloids into two groups and of detecting the presence of more than one component when such components are not members of the same group.

In an effort to find a better method for the separation of the alkaloids, multibuffer paper chromatography[37] was investigated. This procedure had been reported to give good separation of substances which could not be separated by ordinary paper chromatography,[37] and it had even been employed to separate oxyacanthine and repandine, two diasterioisomeric biscoclaurine alkaloids.[86] For multibuffer paper chromatography, the sample is applied at one end of a strip of paper containing a series of buffered zones of increasing acidity. The sample is washed through the zones with an organic solvent until it is retained by a buffer of sufficiently high acidity. The preparation of the paper strips is quite tedious and a new strip is required for each sample.

Direct development of the paper strips with iodine vapor was not effective and iodoplatinate reagent was used instead. The spots were V-shaped, had long tails, and were of low intensity. Thus the presence of small impurities, especially when these followed major components, might not have been detected.

The various greenheart alkaloids gave values ranging from 5.8 to 5.0 for the pH of the buffer zones at which the spots were stopped. Alkaloids I and J each gave two distinct spots which in both cases were at values of 5.6 and 5.2, and they were thus again shown to be mixtures. The values obtained were not related definitely to the group to which the alkaloids belonged nor to the distribution coefficients of the alkaloids. The reproducibility of the method was not as good as might be desired, and sometimes the alkaloids were stopped in the zone previous to the one which is listed in Table IX.

Countercurrent distribution is often an excellent method for determining the purity of a natural product. Alkaloid E hydrochloride was subjected to counter-current distribution in a 54-tube stainless-steel Craig apparatus, employing 0.5 $\underline{M}$ acetate buffer, pH 4.2, and chloroform. At the end of the run the aqueous layers were made alkaline, the alkaloid was extracted into the chloroform layers, and the absorbances of these layers were determined. The resulting distribution pattern is shown in Figure 8. Although the curve is broader than the curve calculated for a pure compound, only one major component is indicated. The deviation from the theoretical curve[31] and the low additional peak at the beginning of the curve could be due to decomposition products formed during the handling of the material. It is possible that better results might be obtained with an all-glass apparatus which could be kept under an inert atmosphere.

The infrared spectra of the alkaloid hydrochlorides in potassium bromide disks again appeared to place the alkaloids into two groups. In the region from 650 to 4000 cm^{-1}, the spectra of rodiasine dimethochloride and of the hydrochlorides of alkaloids C, D, E, and I all resembled each other. The spectra of alkaloids B, F, G, and H also resembled each other. Alkaloid J hydrochloride appeared to occupy an intermediate position. The spectra of rodiasine dimethochloride and of alkaloid I methochloride were indistinguishable from each other, and the spectra of alkaloids B and G were likewise indistinguishable.

Additional information was obtained from the infrared absorption peaks attributable to the phenolic groups of the alkaloids. These peaks in the region near 3μ could not be studied in potassium bromide pellets because of strong inter-ference in this region. This interference by a peak at about 2.9μ was greater when longer mixing times were employed in the preparation of the pellet, and it could not be eliminated. Such interference has been observed also by others.[87, 88] The region of interest could be studied better with chloroform solutions of the free alkaloids, in a Beckman DK-2 spectrophotometer.

The phenolic absorption peaks of chloroform solutions of alkaloids B, F, and G showed maxima at 3545 to 3555 cm^{-1}. Alkaloids C, D, and E gave broader peaks with maxima at 3360 to 3385 cm^{-1}. Somewhat lower peaks in the same general region were shown by alkaloid I at 3380 cm^{-1} and by alkaloid J at 3350 cm^{-1}.

The infrared spectrum of a chloroform solution of alkaloid H had no peak attributable to a phenolic group. It did have a weak peak at 3300 cm^{-1} which could be attributed to the presence of a secondary amino group. Alkaloids B and G gave the same peak but in the spectrum of alkaloid E this peak was lacking or was masked by the broad phenolic peak. In hexachlorobutadiene mulls, both alkaloid G and alkaloid E gave weak peaks at 3285 cm^{-1} and 3319 cm^{-1}, respectively. Very weak peaks of secondary amino groups have been observed for other alkaloids.[89]

The sharp peaks given by alkaloids B, F, and G in the region near 3550 cm^{-1} appear to be characteristic of phenols substituted by ether groups in the ortho position. The broader, lower bands shown by the other alkaloids, except for alkaloid H, appear to be characteristic of phenolic groups with strong internal hydrogen bonding. Flett,[90] who assigned these positions, studied hydroxy compounds in carbon tetrachloride solution. The relative positions of the peaks obtained with chloroform and with carbon tetrachloride solutions of the alkaloids were similar, although the actual positions were at somewhat lower wave numbers when chloroform was employed. The effect of the solvent was greater for alkaloid E which has strong internal bonding (3360 versus 3451 cm^{-1}), than for alkaloid G which does not have internal hydrogen bonding (3555 versus 3560 cm^{-1}).

A phenolic biscoclaurine alkaloid with one diphenyl ether linkage is a long flexible molecule, which in solution could easily give rise to strong hydrogen bonding from the phenolic hydrogen to the oxygen of one of the methoxyl groups. Such a possible bonding could be visualized in molecules represented by formulas III to VI, page 74, but with one of the methoxyl groups replaced by a phenolic group, or by inspection of models of such compounds, such as dauricine, Figure 9. On the other hand, phenolic biscoclaurine alkaloids with two diphenyl ether linkages are not sufficiently flexible to give internal hydrogen bonding. This greater rigidity can be visualized by inspecting formulas VII to X, page 81, or a model such as that of formula XV, shown in Figure 10.

It thus becomes possible to classify the alkaloids into two groups: Group A which could consist of biscoclaurine alkaloids with one diphenyl ether linkage, and Group B which could consist of biscoclaurine alkaloids with two diphenyl ether linkages. In Table IX the alkaloids have been so grouped and names have been assigned to the alkaloids.

In the naming of the alkaloids several relationships are implied. Alkaloids B and G are very similar and presumably very closely related. They were named ocoteamine and isoocoteamine, respectively. The relationship between rodiasine, norrodiasine, and isorodiasine was evidenced by the methanol-insoluble methiodides obtained from these alkaloids and is further discussed below, in Section IV. G.

The alkaloids in Group A all have specific rotations below 100°. Those in Group B have absolute specific rotation values close to or above 200°. It is quite possible that the restriction to free rotation imposed by a second diphenyl ether linkage of a biscoclaurine alkaloid could give rise to the high rotation of the alkaloids in Group B. Tomita and co-workers have reduced biscoclaurine alkaloids with two diphenyl ether linkages to the related biscoclaurine alkaloids with one diphenyl ether linkage.[91] They noted in these cases a decided decrease in specific rotation. They further noted that the high specific rotations were common for alkaloids related to that shown in formula IX, and that the lower rotations were equally typical for alkaloids related to formula III.
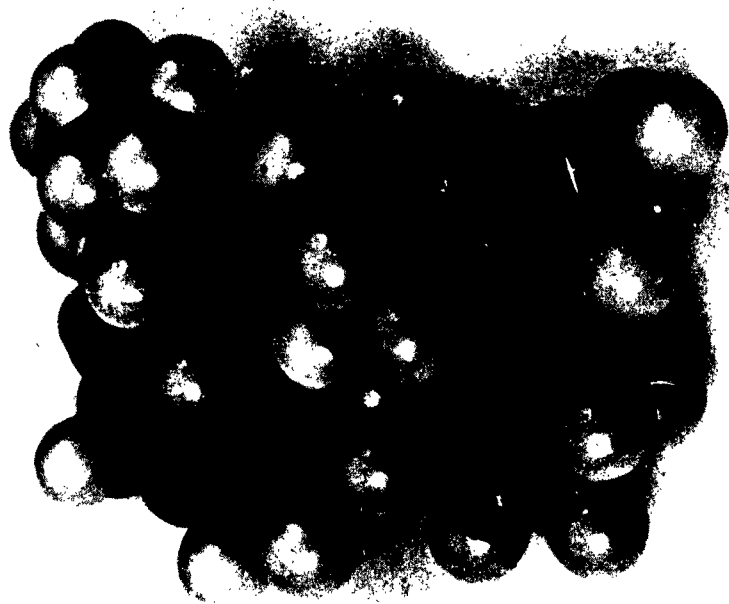
Figure 9. Model of dauricine.

Figure 10. Model of formula XV.

85

Table IX. Properties of Alkaloids From Greenheart

Name of Alkaloid and Original Designation	$[\alpha]_D^o$, a/	K b/	R_f c/	MBPC d/	Phenolic Peak, cm^{-1}	Millon's Test e/	Functional Groups f/ OH	Functional Groups f/ NH
Group A								
Dirosine, E	+97	2.8	0.40	5.4	3360	w	1	1
Rodiasine, D	+74	0.7	0.46	5.0	3385	–	1	0
Norrodiasine, C	+74	2.3	0.42	5.2	3365	?	(1)	(1)
Isorodiasine, i g/	+73	0.6	(0.48)	(5.2)	(3380)	(–)	(1)	(0)
Group B								
Ocoteamine, B	+250	10.5	0.33	5.6	3555	+	(1)	(1)
Isoocoteamine, G	+236	9.1	0.31	5.6	3555	+	1	1
Otocamine, H	+268	0.4	0.34	5.2	none	–	(0)	(1)
Demerarine, F	–181	11.5	0.33	5.8	3545	+	1	1
Ocodemerine, j g/	–170	0.5	(0.33)	(5.6)	(none)	(–)	(0)	(1)
Mixtures								
I	+148	0.5	0.35 / 0.48	5.6 / 5.2	3380	–	1/2	1/2
J	–38	1.4	0.33 / 0.43	5.6 / 5.2	3350	w		

a/ Specific rotation of the hydrochloride (c 1.0 in water).

b/ Distribution coefficient of the hydrochloride in 0.5 M acetate buffer, pH 4.17–chloroform.

c/ For amyl alcohol, pyridine, water (110:110:90) on buffered paper, as discussed in Section III.J.4. Values in parentheses in this column, and in other portions of the table, were not obtained directly but are indicated by various considerations, as explained in the text.

d/ Multibuffered paper chromatography, pH of furthest advance, as discussed in Section III.J.4.

e/ + = positive, – = negative, w = weak with positive confirmatory test, ? = questionable, as discussed in Section III.J.7b.

f/ The number of functional groups per molecule are deduced from the absorption peaks of the acetylated alkaloids, as listed in Section III.J.8.

g/ Originally obtained as a component of this mixture of hydrochlorides.

The far ultraviolet spectra of the greenheart alkaloids in the region of 230 to 190 mμ showed very broad peaks in the range of 205 to 195 mμ. These did not appear very characteristic and were not too unlike the peaks of isochondrodendrine hydrochloride and dimethyl-d-tubocurarine chloride at 202 mμ and 200 mμ, respectively. No peak or shoulder was observed at 225 mμ.

The weak 225-mμ peak of dimethyl-d-tubocurarine iodide disappeared and became a shoulder on conversion to the chloride. Isochondrodendrine hydrochloride and tubocurarine chloride gave only shoulders at 225 mμ, whereas potassium iodide itself gave a peak at 225 mμ. It thus appears that the peaks reported for the series of known methiodides in Section IV. B above, and shown in Figure 1, are primarily caused by the iodide ion and that the contribution of the quaternary biscoclaurine ion is minor.

In the near ultraviolet region, from 345 to 245 mμ, the absorption curves of the greenheart alkaloids and their derivatives began to rise at about 300 mμ, reached a peak at about 283 mμ and a minimum near 260 mμ, and showed no other chromophoric groups. Similar curves were obtained for isochondrodendrine and for tubocurarine chloride and their derivatives, and have been reported for other biscoclaurine alkaloids. [35, 73-75]

Differential peaks of the alkaloids versus their hydrochlorides gave peaks at 295 mμ for both dirosine and isoocoteamine, as shown in Figures 6 and 7. Isochondrodendrine and cycleanine did not give this peak but gave lower peaks at 286 mμ and 279 mμ. The significance of the 295-mμ peak is not known, and it has not been determined whether this peak is associated with the secondary amino group.

In alkaline solutions, the alkaloids gave strong bathochromic shifts near 300 mμ. The differential spectra of the phenolate ions versus the free alkaloids again were characteristic for each group of alkaloids. Dirosine and isorodiasine methochloride gave phenolate peaks at 313 mμ and 310 mμ, respectively. Ocoteamine, isoocoteamine, and demararine gave phenolate peaks at 302 mμ. Isochondrodendrine and tubocurarine chloride also gave phenolate peaks near 300 mμ. Phenol gave a phenolate peak at 290 mμ.

The bathochromic shifts of the above alkaloids in Group B (from 281 to 302 mμ) is similar in magnitude to the corresponding shift (from 272 to 290 mμ) observed for the absorption peak of phenol and to those recently reported for a number of simple substituted phenols. [92] The corresponding shifts of the Group A alkaloids are greater than those of the Group B alkaloids. The corresponding average shifts for the Group A alkaloids, for the Group B alkaloids, and for the simple phenols are 28 mμ, 21 mμ, and 18 mμ, respectively. The significance of these differences is not known.

Phenolic groups were shown to be present in all the alkaloid hydrochlorides originally isolated, except for alkaloid H hydrochloride, by conversion of the phenolic alkaloids to the cobalt salts of the corresponding o-nitrosophenols. The Millon's test was found to be positive for all the alkaloid hydrochlorides in Group B, except for alkaloid H hydrochloride. A positive test generally indicates a phenol with a free ortho position,[40] but more complicated interpretations are reported for many alkaloids.[20] Rodiasine gave a negative Millon's test. Alkaloid I gave a negative Millon's test. Dirosine and alkaloid J, which contained dirosine, gave only weakly positive tests. Norrodiasine gave questionable results.

The number of phenolic groups and secondary amino groups present in the alkaloids were determined by acetylating the alkaloids and determining the strengths of the O-acetyl and N-acetyl peaks in the infrared spectra. The acetylation of the Group B alkaloids proceeded smoothly in acetic anhydride at 60°. To obtain complete acetylation of the alkaloids in Group A, it was necessary to add pyridine to the reaction mixture.

All the acetylated derivatives of the pure alkaloids had O-acetyl peaks, at 1765 cm^{-1}, of approximately the same absorbance values of 0.40. For the same derivatives, the N-acetyl peaks, at 1630 cm^{-1}, had absorbances of approximately 0.63 except that O-acetylrodiasine showed no N-acetyl peak. It was assumed that each of these alkaloids had one phenolic group and that, except for rodiasine, each had one secondary amino group. This assumption was confirmed by microanalytical results which showed the presence of only one N-methyl group in the alkaloids giving N-acetyl peaks and which showed the presence of single phenolic groups in dirosine and in isoocoteamine by the difference in methoxyl contents obtained on O-methylation of these alkaloids.

Acetylated alkaloid I gave O-acetyl and N-acetyl peaks having absorbances of 0.25 and 0.40, respectively, which indicated that one-half of a phenolic group and one-half of a secondary amino group was present per molecule of mixture.

Samples of the hydrochlorides of dirosine and of alkaloids D, C, and I were converted to the free bases and were treated with methyl iodide. Dirosine did not give a methanol-insoluble methiodide and was therefore different from rodiasine. Alkaloids C and D each gave a methanol-insoluble methiodide which on recrystallization melted at 320° with decomposition. This indicated that the two alkaloids, C and D, must originally have differed only in the degree of methylation of the amino group. Since alkaloid D was a di-tertiary alkaloid it was designated rodiasine, and since alkaloid C contained one less N-methyl group it was designated norrodiasine. Alkaloid I also gave a methanol-insoluble methiodide. The latter melted at 314° with decomposition and presumably was isorodiasine dimethiodide.

Conversion of alkaloid I methiodide to the methochloride gave isorodiasine dimethochloride, decomposing at 307°, whose infrared spectrum was indistinguishable from that of rodiasine dimethochloride, decomposing at 283°. Thus one of the components of alkaloid I was isorodiasine. The possibility that this material might be a norisorodiasine, with a secondary amino group, was excluded by the fact that it was isolated from the chromatography in the same region where rodiasine was isolated and not from the later fractions which contained norrodiasine.

Dirosine was converted to the free base and was methylated with ethereal diazomethane. Since equivalent-weight determinations indicated that the methylation was not complete, the product again was converted to the free base and again was methylated with diazomethane. The resulting product was purified by recrystallization of the hydrochloride. Isoocoteamine was also converted to the free base and was methylated with ethereal diazomethane. This product likewise was recrystallized as the hydrochloride.

The various alkaloid hydrochlorides and the two O-methyl derivatives were submitted for microanalyses. The results for the alkaloids in Group A were in agreement with the postulated biscoclaurine structures with one diphenyl ether linkage. Dirosine, which contained four methoxyl groups and presumably one phenolic group, gave an O-methyl derivative containing five methoxyl groups. Similarly, the results were in agreement with the postulated biscoclaurine structure with two diphenyl ether linkages for the alkaloids in Group B. Isoocoteamine, which contained three methoxyl groups and presumably one phenolic group, gave an O-methyl derivative containing four methoxyl groups.

Ocoteamine, isoocoteamine, and otocamine, according to microanalytical results, contained one N-methyl group. The other amino groups presumably were secondary amino groups. Otocamine has the four methoxyl groups which would be expected of a nonphenolic alkaloid with two diphenyl ether linkages. Dirosine and norrodiasine similarly contained one N-methyl group and therefore presumably one secondary amino group. Rodiasine itself had two N-methyl groups and therefore presumably no secondary amino groups. The absence of secondary amino groups in rodiasine had been shown by the acetylation experiments, and the presence of one secondary amino group in isoocoteamine, dirosine, and norrodiasine had been indicated by the same experiments.

The alkaloid hydrochlorides, according to analytical results, all contained one or more molecules of water of hydration. The equivalent-weight determinations for the most part were therefore somewhat low, but the errors in the titration and those due to the unaccounted-for water of hydration were such as to cancel out in many instances and to give the correct equivalent weights of the free bases.

Thus, the purified greenheart alkaloids may apparently be divided into the two groups postulated above. Group A may consist of biscoclaurine alkaloids with one diphenyl ether linkage whose completely methylated derivatives may have formulas III to VI, page 74. Group B may consist of biscoclaurine alkaloids with two diphenyl ether linkages whose completely methylated derivatives may have the structures VII to X, page 81. Although the data presented above strongly suggests these relationships, they do not yet prove the existence of biscoclaurine alkaloids.

From the data in Table IX, some further deductions can be made about the compositions of the mixtures I and J. Since alkaloid I contains isorodiasine as shown by the methiodide which it produces, some of the properties obtained for the mixture must be those of isorodiasine and these are so listed (in parentheses). Since isorodiasine is phenolic and is in Group A, the other component present in alkaloid I must be nonphenolic and a member of Group B. If this component is one of the known Group B alkaloids, it can only be otocamine. Since isorodiasine is phenolic its phenolic absorption peak must be at 3380 cm^{-1}. Since alkaloid I did not give a Millon's test, isorodiasine itself must also give a negative Millon's test. The separation of alkaloid I into isorodiasine and otocamine was subsequently accomplished and is discussed in Section IV. G.

Alkaloid J according to its R_f values presumably also contains components from both groups. The component from Group B must be a nonphenolic material because no phenolic peak was detected at 3550 cm^{-1}. Since alkaloid J has a negative rotation it is quite probable that the component from Group B, with the stronger absolute specific rotation, also has a negative rotation. This component must therefore be different from the other alkaloids in Group B and it is designated ocodemerine. Since ocodemerine is a nonphenolic alkaloid it presumably will have a rather low distribution coefficient. If the other component in alkaloid J is a known alkaloid of Group A, it could possibly be dirosine or norrodiasine, both of which have higher distribution coefficients than alkaloid J. The fact that alkaloid J was obtained from fractions 7 and 8 of the chromatography, which also contained dirosine, whereas norrodiasine was obtained from fractions 9 and 10, and also the results of the Millon's tests, point more strongly towards dirosine as the second component in alkaloid J. The separation of alkaloid J into ocodemerine and dirosine was subsequently accomplished and is discussed in Section IV. F.

Seven alkaloids present in greenheart have thus been characterized by the determination of a number of physical and chemical properties, and a number of properties have been determined for two other greenheart alkaloids. Names have been given to these nine alkaloids, and some relationships between them have been established.

E. Ocoteamine, Isoocoteamine, and Otocamine

The properties of ocoteamine and isoocoteamine, originally isolated as alkaloids B and G, were so similar that it was at first assumed that these two alkaloids were the same material. However, there were definite differences in the specific rotations and in the distribution coefficients of the hydrochlorides, and a slight difference in the melting points of the free alkaloids, as shown below. Ocoteamine was, furthermore, much less soluble in chloroform than isoocoteamine.

Property	Ocoteamine	Isoocoteamine
Of Hydrochloride		
$\underline{K}$ (acetate, pH 5.25 – chloroform)	1.91	1.45
$\underline{K}$ (phosphate, pH 6.25 – ethyl acetate)	2.54	1.76
$[\alpha]_D$ ($\underline{c}$ 1.0 in water)	$+250°$	$+236°$
Of Free Alkaloid		
Melting Point	$221.5-222.5°$	$219-220°$
Solubility in Chloroform	<15 mg./ml.	>30 mg./ml.

Ocoteamine and isoocoteamine thus appeared to be two very closely related alkaloids. It further appeared, as was discussed in the previous section, pages 89-90, that they were biscoclaurine alkaloids with two diphenyl ether linkages and that their completely methylated derivates would probably have the structures VII, VIII, IX, or X, or structures which are optical isomers of these.

VII

VIII
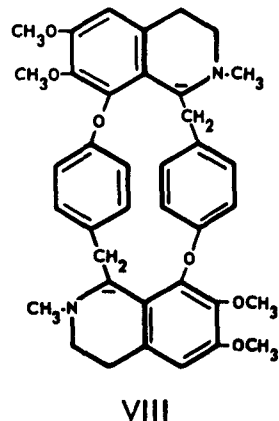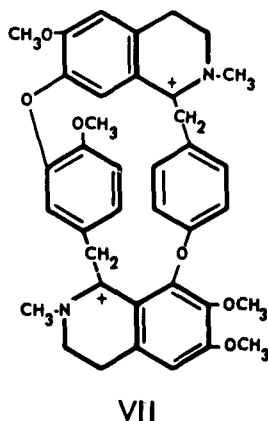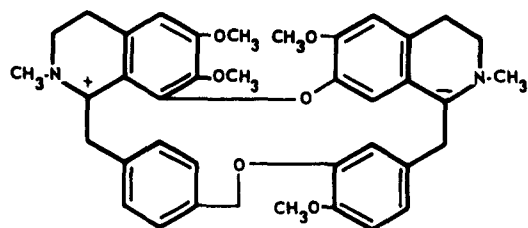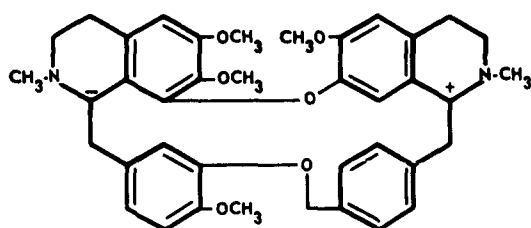
IX   X

Representative alkaloids from each of these four series were available.
Tubocurarine chloride (XI), the quaternary derivative of a diasterioisomer of chon-
drodendrine (I), was available as a commercial U.S.P. preparation. Isochondrodendrine
(II) had been prepared from commercial "bebeerine hydrochloride." Oxyacanthine
(XII) was obtained from commercial oxyacanthine hydrochloride purchased from
Flucka A. G., Buchs, Switzerland. Berbamine (XIII) was obtained from commercial
berbamine hydrochloride also purchased from Flucka A. G.
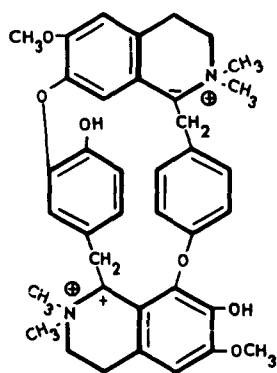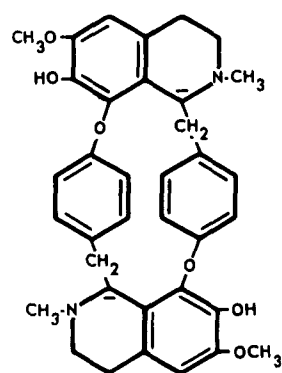
XI   II
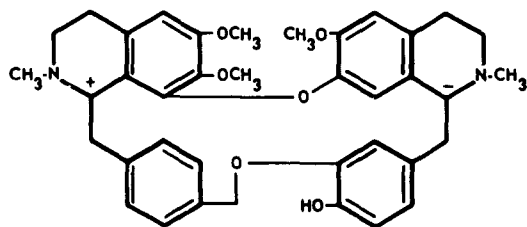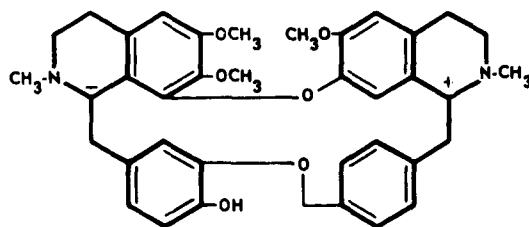
XII   XIII

The infrared spectra of ocoteamine and isoocoteamine, which were identical, were compared with those of the above four model compounds. They were found to be very closely related to that of oxyacanthine. The spectra of ocoteamine and isoocoteamine, however, lacked a peak at approximately 1380 cm^{-1} and a doublet at approximately 1360 cm^{-1} which were present in the spectrum of a chloroform solution of oxyacanthine. The same differences had been noted between the spectra of norrodiasine and rodiasine, which had been shown to have a des-N-methyl relationship. It therefore appeared possible that ocoteamine and isoocoteamine were des-N-methyl derivatives of oxyacanthine or of one of its isomers.

Isoocoteamine was N-methylated with formalin and formic acid[93, 94] and the product was purified by crystallization of the hydrogen sulphate and of the hydrochloride. The N-methylisoocoteamine hydrochloride obtained was indistinguishable from oxyacanthine hydrochloride. N-methylocoteamine was similarly prepared and its hydrochloride was again indistinguishable from that of oxyacanthine. The three alkaloid hydrochlorides had identical infrared spectra and their specific rotations and distribution coefficients in acetate buffer, pH 4. 17 - chloroform were identical, within experimental error, as shown:

Hydrochloride of	$\underline{K}$	$[\alpha]_D$
N-Methylocoteamine	2.53	+186°
N-Methylisoocoteamine	2.59	+187°
Oxyacanthine	2.36	+188°

Ocoteamine and isoocoteamine were therefore des-N-methyl derivatives of oxyacanthine. Two such derivatives are possible and these have the formulas XIV and XV.
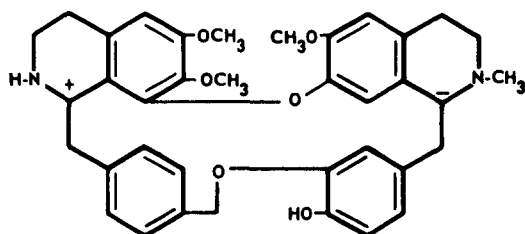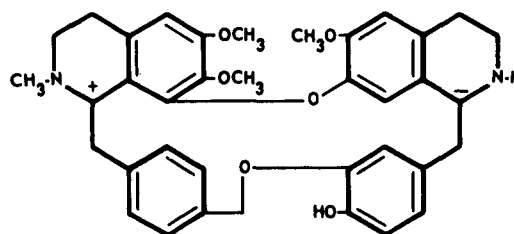

XIV   XV

If ocoteamine and isoocoteamine had the above two structures, it remained to determine the specific structure of each of the two alkaloids. O-methylation of the two structures indicated should give two new and different alkaloids, which have the formulas XVI and XVII. Both of these alkaloids have been described in the literature. Hydroepistephanine-A[60] has formula XVI, and O, O-dimethyltrilobamine,[44] also known as O, O-dimethyldaphnoline,[45] has the formula XVII.

XVI   XVII
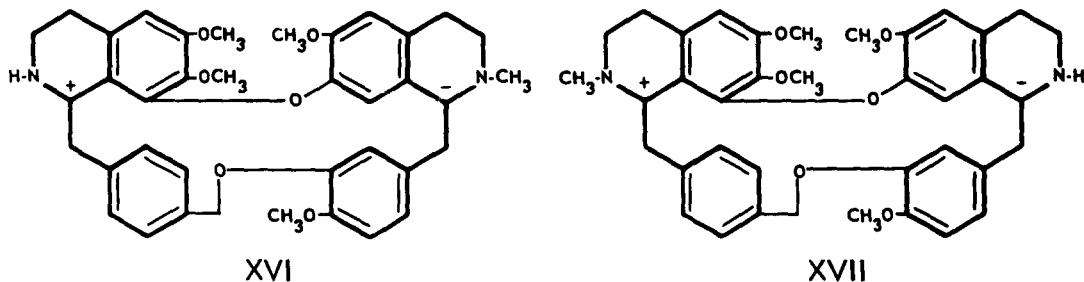
Ocoteamine and isoocoteamine were methylated with diazomethane and the hydrochlorides of the resultant products were purified by recrystallization to constant specific rotation. The two products differed from each other as was shown by the different distribution coefficients and the different specific rotations. The values obtained and the values reported for the hydrochlorides of XVI and XVII are listed in Table X.

O-Methylocoteamine hydrochloride had a higher specific rotation than O-methylisoocoteamine hydrochloride. It therefore seemed possible that these two derivatives might be the hydrochlorides of hydroepistephanine-A (XVI) and of O, O-dimethyltrilobamine (XVII), respectively. The formula of ocoteamine would then be XIV and the formula of isoocoteamine would be XV.

The specific rotations of the hydrochlorides of O-methylocoteamine and O-methylisoocoteamine (+279° and +258°, respectively) were actually considerably lower than those of the hydrochlorides of hydroepistephanine-A and O, O-dimethyltrilobamine (+298° and +272°, respectively). These lower-than-expected specific rotations could have been caused by the presence of impurities.

Small quantities of impurities which might be difficult to separate could have been introduced in the O-methylation of ocoteamine and isoocoteamine. The O-methylation of the various alkaloids with diazomethane in all instances gave yields of approximately 50% or less, and other authors also have reported comparatively low yields of O-methylation products from biscoclaurine alkaloids. (It is of interest that O-methyloxyacanthine obtained by O-methylation with diazomethane could not be obtained in a crystalline form,[95] whereas the same alkaloid obtained from another source could be crystallized.)

Table X. Hydrochlorides of Des-N-methyl-O-methyloxyacanthines

Hydrochloride of	$\underline{K}$[a]	Specific Rotation[b]			$\underline{R}_f$[c]
		$\underline{c}$ 1.00	$\underline{c}$ 0.50	$\underline{c}$ 0.25	
Literature Values[d]					
Hydroepistephanine-A	—	—	—	+298°	—
O, O-Dimethyltrilobamine	—	—	+272°	—	—
Experimental Values					
O-Methylocoteamine	0.47	+263°	+268°	+279°	0.62
O-Methylisoocoteamine	0.33	+256°	+258°	—	0.62
Hydroepistephanine-A	1.03	—	—	+300°	0.58
Otocamine	0.39	+268°	+277°	+278°	0.62

a/ Distribution coefficient for 0.5 $\underline{M}$ acetate buffer, pH 4. 17 - chloroform.

b/ $[\alpha]_D$ at the three concentrations indicated; concentration in grams per decaliter of water; temperatures of 20° and 31°, respectively, for the two literature values, and 25-26° for the experimental determinations.

c/ For the free alkaloids with amyl alcohol, pyridine, water (110:110:90) on buffered paper.

d/ References 44 and 60.

    There was thus the one possibility that ocoteamine and isoocoteamine were XIV and XV, respectively. A second possibility was that they were essentially the same alkaloid and that one of the preparations, originally obtained as alkaloids B and G, contained an impurity. Such an impurity would have to be one which cannot be removed by recrystallization of the alkaloid hydrochloride or of the free alkaloid from acetone or methyl alcohol. It would have to be an impurity which is not removed in the O-methylation procedure but is removed during the N-methylation procedure. If ocoteamine and isoocoteamine actually should differ only because of the presence of an impurity, then O-methylocoteamine and O-methylisoocoteamine would differ similarly.

    To determine the relationship of O-methylocoteamine and O-methylisoocoteamine to XVI and XVII, it was desirable to obtain these known compounds from other sources and to make direct comparisons. Hydroepistephanine-A was prepared by reduction of

a sample of epistephanine (XVIII) which was kindly supplied by Dr. Yasuo Watanabe of the First College of Pharmacy, Fukuoka City, through the good offices of Dr. Masao Tomita of the University of Kyoto. The reduction of epistephanine by zinc and sulphuric acid is reported to be stereospecific.[60] The hydrochloride of hydroepistephanine-A (XVI) thus prepared had essentially the specific rotation reported in the literature. It was not possible to obtain samples of O, O-dimethyltrilobamine (or of O, O-dimethyldaphnoline).

XVIII   XVI
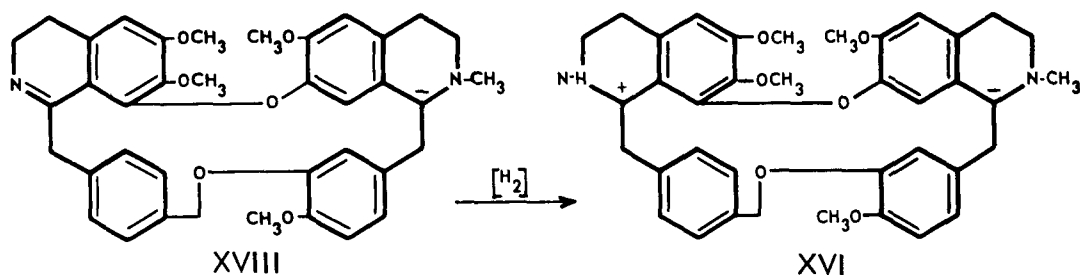
Hydroepistephanine-A hydrochloride was found to have a distribution coefficient of 1.03 and an R_f of 0.58, as shown in Table X. Thus, neither O-methylocoteamine nor O-methylisoocoteamine, which have distribution coefficients of about 0.4, R_f values of 0.62, and identical infrared spectra different from that of hydroepistephanine-A, can have structure XVI. They must therefore both have the structure XVII, which is the only other alternative.

Ocoteamine and isoocoteamine must therefore both have structure XV. The phenolic group of both alkaloids can be in no other position than that indicated, since it must be in the same position as the phenolic group of oxyacanthine (XII). The slight differences in the properties of ocoteamine and isoocoteamine and of their O-methyl derivatives are presumably due to impurities as discussed above.

With the available analytical methods, the presence or absence of impurities in ocoteamine or isoocoteamine, or in their derivatives, could not be ascertained. In paper partition chromatography and in multibuffer paper chromatography, weak second spots were on occasion obtained for both alkaloids or for their derivatives. However, these evidences of impurities were not consistent and could have been due to impurities formed in the handling of the alkaloids. With the newer method of thin-layer chromatography,[96] it might be possible to determine whether ocoteamine or isoocoteamine is the purer of these alkaloids. The higher melting point and lower solubility in chloroform, and the higher specific rotation of the alkaloid hydrochloride and of its O-methyl derivative (the latter being closer to that reported for O, O-dimethyltrilobamine), suggest that ocoteamine is purer than isoocoteamine.

O, O-Dimethyltrilobamine (or O, O-dimethyldaphnoline, XVII) has been cleaved by reduction with sodium in liquid ammonia to give XIX and XX.[44, 45] The d-armepavine (XIX) was isolated as the oxalate. The second base (XX) was methylated with diazomethane to O, O-dimethylcoclaurine (XXI),[45] or was identified by conversion to the fully methylated methiodide, l-O-methylarmepavine methiodide.[44]

XVII   XIX   XX

XXI
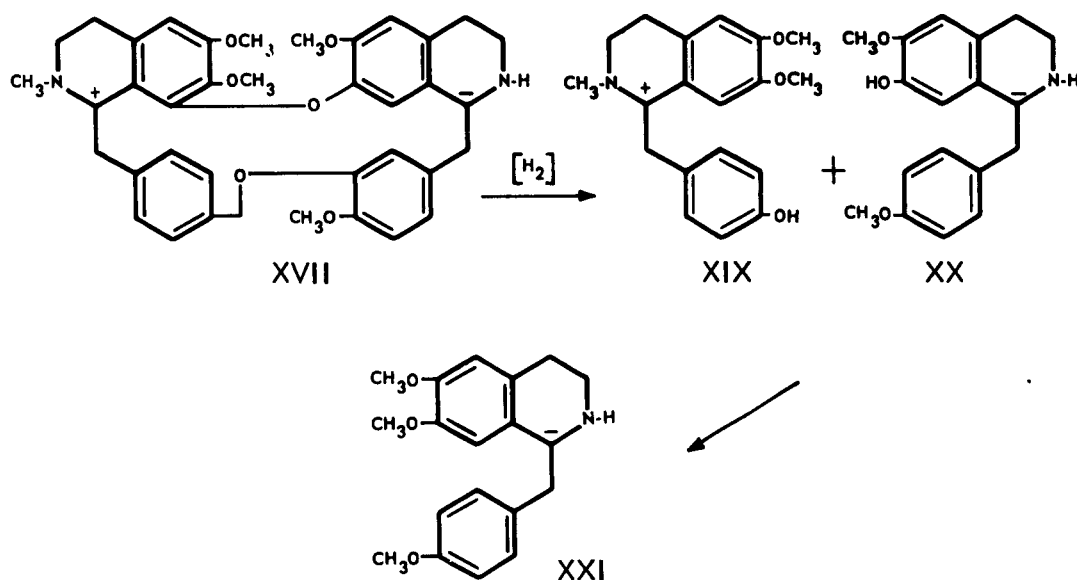
On the other hand, hydroepistephanine-A (XVI, or XVII with the secondary amino group and the tertiary amino group interchanged) would not give armepavine (XIX). It would instead give the coclaurine derivatives analogous to XIX and XX, but with the secondary amino group and the tertiary amino group interchanged.

The cleavage of O-methylisoocoteamine by reduction with sodium in liquid ammonia gave d-armepavine (XIX), which was isolated as the oxalate. The starting material could therefore have the structure XVII but could not have the structure XVI. These results constitute further evidence that ocoteamine (and isoocoteamine) must have the structure XV, Figure 10, rather than structure XIV.

A consideration of the properties of otocamine, listed in Table IX, indicated the possibility that this alkaloid might be the O-methyl derivative of ocoteamine or of isoocoteamine. The specific rotation and the distribution coefficient of otocamine hydrochloride were therefore compared with those of the hydrochlorides of these derivatives. The different distribution coefficients and specific rotations, as listed in Table X, indicated that the three hydrochlorides differed from each other, but that the differences were slight.

97

The possibility of a relationship between the otocamine and berbamine was considered. It was noted, however, that the infrared spectrum of otocamine hydrochloride was quite different from that of berbamine hydrochloride and was considerably more similar to that of oxyacanthine hydrochloride. Moreover, the spectrum was almost indistinguishable from those of the hydrochlorides of O-methylocoteamine and O-methy' ocoteamine.

Otocamine was N-methylated with formalin and formic acid. The N-methylotocamine hydrochloride obtained had an infrared spectrum and a specific rotation which were indistinguishable from those of O-methyloxyacanthine hydrochloride. Otocamine, therefore, was a des-N-methyl derivative of O-methyloxyacanthine (IX) and could only have the structures XVI or XVII. These alkaloids are hydroepistephanine-A and O, O-dimethyltrilobamine.

Otocamine was different from hydroepistephanine-A as shown by the considerable difference in the specific rotations and by the smaller difference in R_f values shown in Table X, and by considerable differences in the infrared spectra of the hydrochlorides as shown in Appendix B.

Otocamine therefore must be the same as O, O-dimethyltrilobamine. The specific rotation of +277° of otocamine hydrochloride was similar to the specific rotation of +272° reported for O, O-dimethyltrilobamine. The difference in values can be considered within experimental error for measurements by different investigators at slightly different temperatures.

Otocamine thus has been shown to have the structure XVII and to be the same as O, O-dimethyltrilobamine (or O, O-dimethyldaphnoline), which had not been obtained previously as a naturally occurring alkaloid. Otocamine therefore must be the same as O-methylocoteamine.

While the above work was in progress, a communication[97] on alkaloids from greenheart was published by Dr. M. F. Grundon of the Queen's University of Belfast, and a paper was published by Grundon and McGarvey.[46] This paper described an alkaloid which was called sepeerine. A structure of XIV or XV was proposed for this alkaloid, but the properties given for this compound were such that no identity with ocoteamine or isoocoteamine could be established. A sample of sepeerine was obtained from Dr. Grundon and this material had an infrared spectrum which was indistinguishable from those of ocoteamine and isoocoteamine. Grundon and McGarvey later established the structure XV for sepeerine.[98]

There remained, however, a number of differences in the physical properties of ocoteamine or isoocoteamine and of sepeerine, and in the physical properties of the corresponding hydrochlorides of the O-methyl derivatives. Some of the observed

values are compared below to values reported in the literature.[44,46] The lower
melting point of sepeerine and the low specific rotation of O–methylsepeerine
hydrochloride may be due to the presence of impurities.

Alkaloid	M. P.[a/]	$[\alpha]_D$[b/]	$[\alpha]_D$[c/]
Ocoteamine	222.5°	—	+268°
Isoocoteamine	220°	+392°	+258°
Sepeerine	199°	+391°	+235°
Trilobamine	—	—	+272°

a/ of the alkaloid crystallized from methanol: ocoteamine and isoocoteamine
consisting of platelets and sepeerine consisting of rods.

b/ of the alkaloid in chloroform.

c/ of the hydrochloride of the completely O–methylated derivative in water.
(Further values are given in Table X.)

It thus appears that sepeerine isolated by Grundon is essentially the same as
ocoteamine, but that it possibly contains some impurities. It would seem preferable
to use the name ocoteamine for this alkaloid rather than the name sepeerine. The
name sipeerine had been used previously for the ether–insoluble alkaloids of green–
heart [8] and the use of practically the same designation for a component of the
ether–soluble alkaloids might lead to confusion.

F. Demerarine and Ocodemerine

Demerarine was the second most abundant alkaloid obtained by the chromatography
of the ether–soluble alkaloids of greenheart bark. It was obtained in a yield of almost
7% from the mother liquors of the crystallization of isoocoteamine hydrochloride.

Demerarine was comparatively unstable, and recrystallization of the hydrochloride
from water, or recrystallization of the free alkaloid from acetone, was accompanied
by decomposition, as evidenced by the darkened products obtained. However,
recrystallizations from water, saturated with chloroform and containing very little
hydrochloric acid, gave a colorless crystalline hydrochloride containing chloroform.
A subsequent recrystallization in the absence of chloroform gave colorless, crystalline
demerarine hydrochloride with a specific rotation of -181°. Conversion to the free
alkaloid and recrystallization from methanol gave crystalline demerarine melting
at 223°.

Demerarine hydrochloride in methanol was treated with ethereal diazomethane. A gelatinous hydrochloride with a specific rotation of -155° was obtained.

Demerarine is in Group B of the greenheart alkaloids, as shown in Table IX. Demerarine appears to be very closely related to ocoteamine (and to isoocoteamine). In addition to the similarities shown in Table IX, in the distribution coefficients, the chromatographic behavior, the positions of the phenolic peaks, and the reaction in the Millon's test, there were strong similarities in the ultraviolet spectra of the three alkaloids. Furthermore, the microanalytical results, Table V, indicated the presence of the same functional groups.

Because of the above similarities it appeared likely that the chief differences between the alkaloids were not differences in the general structure, in the type and number of functional groups, or even in the relative positions of these functional groups. Rather, the differences appeared to be in the stereoisomeric relationship of the skeletons and thus in the configuration of the optically active centers.

The absolute specific rotation of demerarine is considerably less than that of ocoteamine (181° and 250°, respectively, for the hydrochlorides). Therefore, it appears very likely that the skeletons have a diastereoisomeric relationship rather than an enantiomorphic relationship.

Fully methylated demerarine would then have a similar diastereoisomeric relationship to fully methylated ocoteamine. The latter is the same as O-methyloxyacanthine (IX). Fully methylated demerarine therefore is very likely to be a diastereoisomer of IX, such as XXII, and is not likely to be the enantiomorph of IX, which is XXIII.

XXII   XXIII
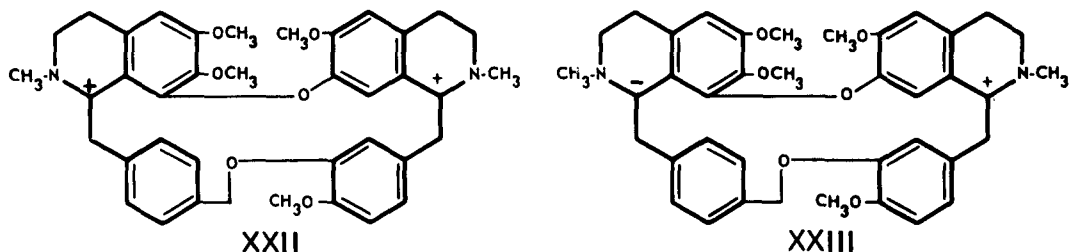
In addition to XXII, there is a second diastereoisomer of IX, which is the enantiomorph of XXII, and which therefore is the same as XXII, except that the optically active centers are both negative. Of these two possibilities, one must have a positive specific rotation and the other must have a negative specific rotation of equal magnitude. Structure XXII is a known alkaloid, O-methylrepandine,

and has a negative specific rotation (of –108° in $\underline{N}$/10 hydrochloric acid).[99] It is therefore very likely that demerarine (having $[\alpha]_D$ –181° for the hydrochloride in water) is related to XXII, and a relationship to the enantiomorph of XXII appears out of consideration.

Because of the similarities between most of the properties of demerarine and ocoteamine, it appears likely that the phenolic groups of the two alkaloids are in the same position (and therefore at the position occupied by the lowest methoxyl group of the above formulas). If fully methylated demararine has structure XXII, then demerarine, which has a free phenolic group and a secondary amino group, is likely to have the structure XXIV or XXV. These structures are the two possible des–N–methyl derivatives of repandine.
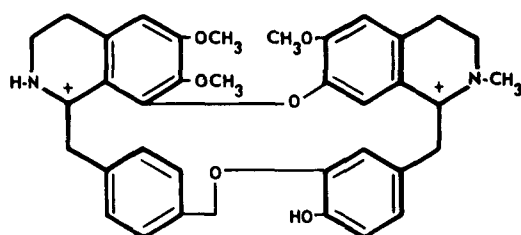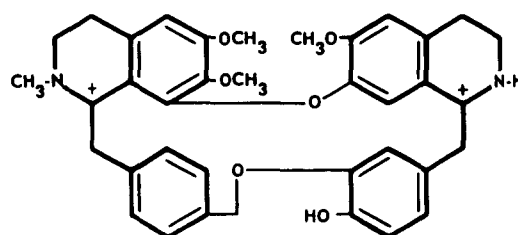
XXIV   XXV

If the above are the possible structures of demerarine, O–methyldemerarine must have structure XXVI or XXVII. The latter structures are diastereoisomers of hydroepistephanine–A (XVI) and of O, O–dimethyltrilobamine (XVII).
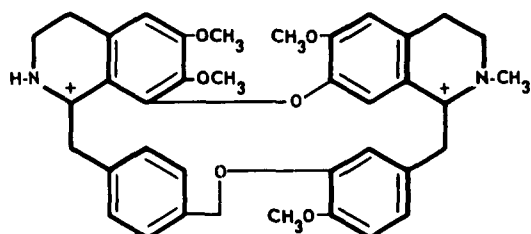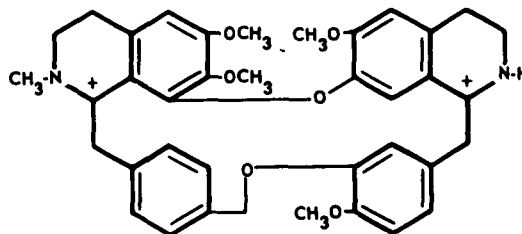
XXVI   XXVII

A differentiation between structures XXVI and XXVII should be possible by cleavage with sodium in liquid ammonia. The former structure (XXVI) should give the O–methylcoclaurine of formula XXVIII and the O, N–dimethylcoclaurine of formula XXIX. On the other hand, the structure XXVII on cleavage with sodium in liquid ammonia should give armepavine (XIX) and the O–methylcoclaurine of formula XXX; the armepavine should crystallize readily from the products as the hydrogen oxalate, and the O–methylcoclaurine (XXX) is the enantiomorph of XX, and should have the opposite optical rotation but the same physical and chemical properties. This differentiation has not been made.

XXVIII   XXIX   XIX   XXX
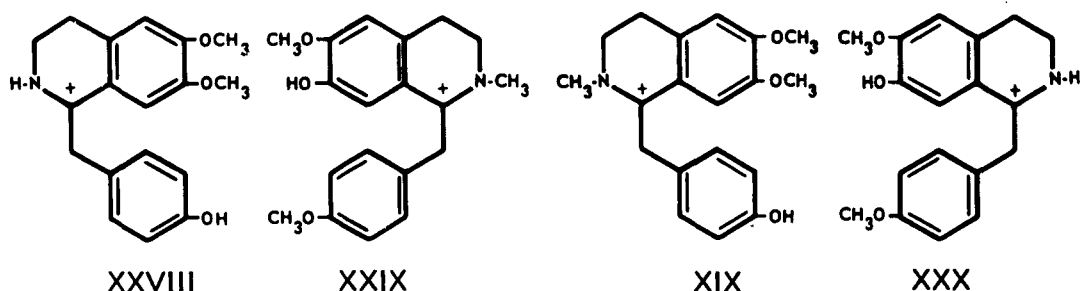
Ocodemerine hydrochloride was isolated from alkaloid J hydrochloride mixture by fractional crystallization. Crystallization of the mixture from alcoholic hydrochloric acid gave dirosine hydrochloride. Recrystallization of the residue from aqueous hydrochloric acid gave ocodemerine hydrochloride with a specific rotation of -170°. The latter recrystallizations were made at 50° to prevent the formation of a gel, which occurred at lower temperatures.

As was discussed in Section IV. D, page 90, it had been expected that ocodemerine would be a nonphenolic alkaloid of Group B and that it would have a negative specific rotation. Subsequent data, including microanalytical results, listed in Table V, and other properties, listed in Table IX, agreed with this postulation. The microanalyses were in agreement with the calculated values for a biscoclaurine alkaloid with two diphenyl ether linkages, four methoxyl groups, and one N-methyl group. The distribution coefficient was in the same range as that of otocamine, which has the same functional groups.

The specific rotation of ocodemerine hydrochloride was very similar to that of demerarine hydrochloride and a relationship between the two alkaloids is suggested. It thus appears quite likely that ocodemerine is the O-methyl derivative of demerarine. However, this relationship has not yet been established. (Treatment of demerarine with diazomethane had given a gelatinous hydrochloride with a specific rotation of -155°, but this product apparently was not pure.)

It appears quite probable, therefore, that ocodemerine is a des-N-methyl-O-methylrepandine and thus has the formula XXVI or XXVII. If demerarine is XXIV, it is most likely that ocodemerine is XXVI; if demerarine is XXV, it is most likely that ocodemerine is XXVII.

G. Rodiasine, Isorodiasine, and Norrodiasine

Rodiasine dimethiodide had been the first pure alkaloid obtained from greenheart.[71] It was produced by N-methylation with methyl iodide of the di-tertiary precursor, rodiasine, or of a des-N-methyl derivative of this alkaloid.

Alkaloid D hydrochloride, on conversion to the free base and treatment with methyl
iodide, gave rodiasine dimethiodide. Since alkaloid D contained two N–methyl
groups and no secondary amino group, it was rodiasine. Rodiasine, containing
four methoxyl groups, was converted to O–methylrodiasine, containing five methoxyl
groups. A C_{38} rather than a C_{19} formula, and the presence of one phenolic group
in rodiasine, were thus established.

Isorodiasine was shown to be present in alkaloid I hydrochloride. Reaction of
alkaloid I in methanol with methyl iodide gave the methanol–insoluble isorodiasine
dimethiodide, melting with decomposition at 314°. This methiodide was converted
to the methochloride, m.p. 307° dec., which had an infrared spectrum identical
with that of rodiasine dimethochloride. The corresponding melting points of rodiasine
dimethiodide and dimethochloride were different, whereas the melting points of
rodiasine (W) methiodide and of the isorodiasine dimethochloride obtained from it
were essentially the same as shown below:

Alkaloid	Methiodide	Methochloride
Alkaloid I	314°	307°
Rodiasine	321°	286°
Rodiasine (W)	314°	—
Isorodiasine	—	309°

Isorodiasine must be a di–tertiary alkaloid, as was discussed in Section IV. D,
page 89. Isorodiasine and rodiasine must have a phenolic group and a methoxyl
group interchanged, because, as was discussed in Section IV. A, page 74, a similar
relationship exists in the respective dimethiodides.

Isorodiasine is a phenolic alkaloid and the other alkaloid present in alkaloid I
is nonphenolic. It presumably is otocamine, as was discussed in Section IV. D.

A separation of the components of alkaloid I on the basis of the phenolic group
was attempted. A sample of alkaloid hydrochlorides from another source, bark (G)
rather than bark (G2), apparently was the same as alkaloid I hydrochloride. The free
alkaloids from this sample were dissolved in benzene and extracted with sodium
hydroxide solution. However, only a negligible amount of material was dissolved
in the sodium hydroxide solution and practically all the material remained in the
benzene. Extraction of a benzene solution with Claisen alkali gave similar results.
The phenolic group which was present was therefore very kryptophenolic.

On recovering the original alkaloid hydrochloride, two types of crystals were obtained: very fine, long needles and shorter, thicker rods. Careful fractional crystallization gave rods of otocamine hydrochloride, with a specific rotation of +268°, and long hairlike needles with a specific rotation of +77°. A sample of the latter hydrochloride was converted to a methanol-insoluble methiodide which melted at 320° with decomposition. The fine, hairlike needles were therefore rodiasine. It thus was evident that either isorodiasine hydrochloride or rodiasine hydrochloride can readily crystallize together with otocamine hydrochloride.

A small sample of the original alkaloid I hydrochloride, which previously had given isorodiasine methiodide and therefore definitely contained isorodiasine, was still available. Careful fractional crystallization of this material gave small rods of otocamine hydrochloride and hairlike needles of isorodiasine hydrochloride having a specific rotation of +73°.

The specific rotation of isorodiasine hydrochloride was practically indistinguishable from that of rodiasine hydrochloride. The distribution coefficients of freshly recrystallized samples of the two hydrochlorides were also indistinguishable, as were the infrared spectra of the corresponding methochlorides. The only detectable differences in properties thus remain the melting points of the methiodides and of the methochlorides. As was mentioned above, the structural differences must be an interchange of the phenolic group with one of the methoxyl groups.

Norrodiasine is a des-N-methyl derivative of rodiasine. The formation of rodiasine dimethiodide from this alkaloid on treatment with methyl iodide showed that norrodiasine and rodiasine were identical except for the degree of methylation of the amino groups. The presence of only one N-methyl group was shown by microanalytical results and the presence of one secondary amino group was shown by the strength of the N-acetyl peak of acetylated norrodiasine as discussed in Section IV. D. Further structure proofs for norrodiasine will most likely hinge on structure proofs for rodiasine.

O-Methylrodiasine appeared to be a biscoclaurine alkaloid with one diphenyl ether linkage and with the empirical formula $C_{39}H_{46}O_6N_2$. The reasons for this probable structure were discussed in Sections IV. B and D, but such a structure has not been proven. The most probable structures for such an alkaloid are III, IV, V, and VI. Each of these structures has two optically active centers and can therefore exist in four optically isomeric forms.
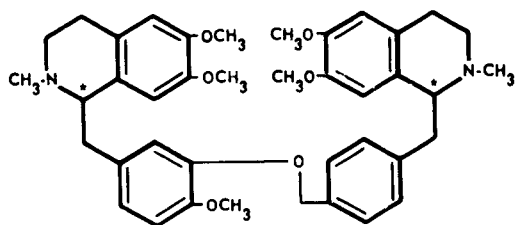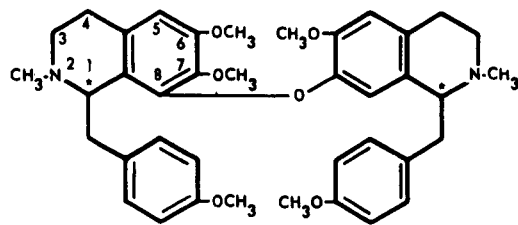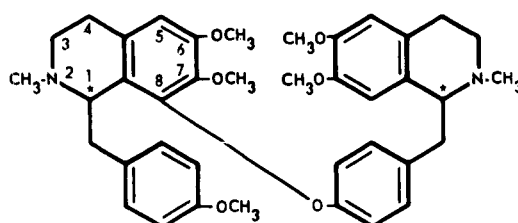
III IV
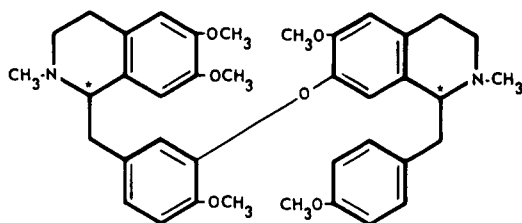
V VI

Two alkaloids of structure III are known. These are O-methyldauricine (XXXI)[100] and O-methylberbamunine (XXXII).[101] The methiodides of these two alkaloids both have melting points of approximately 182° and their respective specific rotations are −151°,[102] and 0°.[101] Since the other two possible isomers having structure III are the enantiomorphs of those just mentioned, they must have the same melting points and equal but opposite specific rotations. Therefore none of these structures III can be the same as O-methylrodiasine, whose methiodide has a melting point of 304° and a specific rotation of +50°.
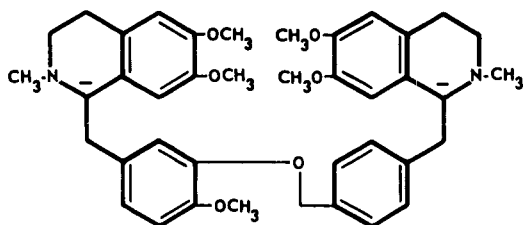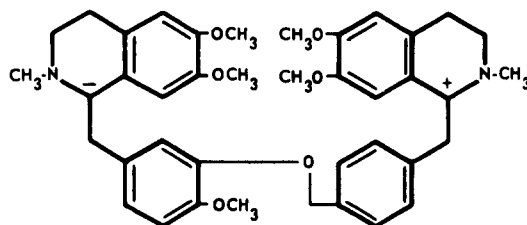
XXXI XXXII

In the O-methylrodiasine skeleton there must be two potential phenolic positions which would give negative Millon's tests. One of these would be the phenolic group of rodiasine, the other the phenolic group of isorodiasine. Ordinarily, the Millon's test is considered positive for phenols with free ortho positions.[40] However, King[20] has found that for biscoclaurine alkaloids and a number of related alkaloids, negative tests are obtained for many phenols with free ortho positions.

In accordance with deductions made by King, phenolic groups in the benzyl portions of formulas IV, V, and VI would give positive Millon's tests. A positive test would be given also by a phenolic group ortho to both a diphenyl ether linkage and a methyl ether linkage, as in position 7 of the left halves of the structures IV and VI. Since rodiasine gives a negative Millon's test, none of these positions should be the location of the phenolic group of rodiasine. (The interpretation of this test is not sufficiently definite to completely rule out these positions. Recently the alkaloid fangchinoline, which has a phenolic group in a position equivalent to the positions 7, as indicated in formula IV or VI, nevertheless has been found to give a negative Millon's reaction.)[45]

Phenolic groups replacing any of the other methoxyl groups of the above formulas (two positions in formula IV, three in V, and three in VI) would give negative Millon's tests. Each of these formulas would thus have two or more such potential sites for the phenolic groups of rodiasine and isorodiasine, and the results of the Millon's tests would not rule out any of these structures.

If O–methylrodiasine had structures IV, V, or VI, a further distinction between the correct structures should be possible by identification of the products of the reduction of O–methylrodiasine with sodium in liquid ammonia. This reduction normally cleaves biscoclaurine alkaloids into two coclaurine derivatives. By this cleavage, O–methyldauricine (XXXI) gave l–O–methylarmepavine (XXXIII) and l–armepavine (XXXIV). Structure VI on reduction with sodium in liquid ammonia should give the same two products, or their enantiomorphs. The reduction of structures IV and V, on the other hand, should give O–methylarmepavine (XXXIII, or its enantiomorph) and an O, N–dimethylcoclaurine (XXIX, or its enantiomorph). In this Laboratory the reductive cleavage with sodium in liquid ammonia was successfully carried out with cycleanine (VIII) to give a single phenolic cleavage product (XXXIV), [103] with isotetrandine (X) to give a phenolic (XXXV) and a nonphenolic (XXXIII) product, [47] and with otocamine (or O–methylisoocoteamine, XVII) to give two phenolic products (XIX and XX).

XXXIII   XXXIV   XXIX   XXXV
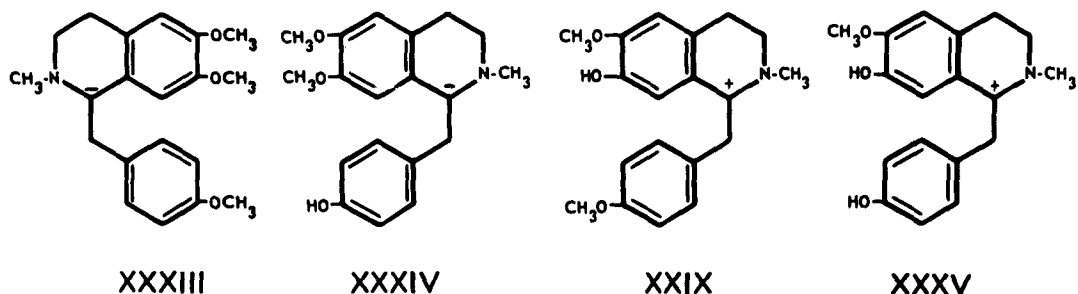
O-Methylrodiasine was reduced with sodium in liquid ammonia. The reaction mixture gave no phenolic product and the nonphenolic product which was obtained was different from the starting material. This result was not expected from any of the structures IV, V, or VI. Two possibilities exist: (1) the reduction for some unknown reason proceeded in an unexpected manner; or (2) O-methylrodiasine does not have any of the structures which on the basis of previous reasoning appeared to be the most logical.

Grundon[46, 97] had obtained an alkaloid from greenheart bark which he called rodiasine because it gave a methanol-insoluble methiodide that was presumed to be the same as rodiasine dimethiodide. A small sample of rodiasine which Dr. Grundon supplied for infrared spectral comparisons was lost because the container broke in transit, and no other sample has been received. From the data reported, it is difficult to determine whether Grundon's sample was rodiasine, isorodiasine, or an incompletely purified material containing either of the alkaloids. Some physical properties of these alkaloids and their methiodides (including specific rotations in chloroform and water, respectively) are as follows:

Alkaloid	Original Alkaloid		Methiodide	
	M. P.	$[\alpha]_D$	M. P.	$[\alpha]_D$
Rodiasine	$211°$	$+153°$	$321°$	$+68°$
Isorodiasine	—	—	$314°$	$+68°$
Grundon's Alkaloid	$195°$	$+134°$	$295°$	$+73°$

Grundon's melting point for both the original alkaloid, rodiasine, and for the methiodide derivative are considerably lower than the values found in this Laboratory. A similar discrepancy had been noted in the comparison of sepeerine with ocoteamine and isoocoteamine. These differences may have been caused by different experimental techniques; for example, the capillary tubing in which the sample was melted might not have been evacuated. However, the specific rotation of Grundon's alkaloid differs appreciably from that of rodiasine, and the specific rotation of +85° reported for Grundon's O-methyl derivative is considerably lower than the specific rotation of +96° of O-methylrodiasine. Grundon reported the crystallization of O-methylrodiasine from ether, but crystallization from ether was not successful in this Laboratory. (A sample of rodiasine dimethiodide was supplied to Dr. Grundon, but no results are available for any comparisons which may have been made.)

H. Dirosine

The most abundant alkaloid isolated from the ether-soluble alkaloids of greenheart bark was dirosine hydrochloride. It was obtained in an 11% yield from the gradient elution chromatography, as alkaloid E hydrochloride. The equivalent weights of alkaloid E hydrochloride and of its O-methyl derivative had indicated the presence of two phenolic groups in alkaloid E and the possibility that it was a biscoclaurine alkaloid with one diphenyl ether linkage and two N-methyl groups. [104] Subsequent microanalytical results showed that the results of the equivalent-weight determinations were misleading.

Microanalyses of dirosine hydrochloride and of O-methyldirosine hydrochloride pointed to a formula $C_{37}H_{40}O_6N_2$ with four methoxyl groups, one N-methyl group, and one free phenolic group for dirosine. Infrared spectra of dirosine and of its acetylated derivative showed the presence of a secondary amino group and the presence of a phenolic group which exhibits internal association. This data is not inconsistent with a biscoclaurine structure having one diphenyl ether linkage.

Dirosine crystallized very readily from methanol but had a broad melting range near 180°, which was not sharpened by recrystallization from ethanol. The alkaloid appeared to consist of a single component and showed a single spot in both paper chromatography and multibuffer paper chromatography. A 54-plate countercurrent distribution of dirosine gave only a single peak, as shown in Figure 8, but the peak was somewhat broader than the theoretical peak. This broadening of the peak and a very weak secondary peak could be due to decomposition which took place during the running of the countercurrent distribution.

Dirosine decomposed rather readily, especially in solution. When a small amount of dirosine was partially dissolved in ether, and the ether was allowed to evaporate, an oily residue was produced together with the remaining crystals. The ultraviolet spectrum of dirosine hydrochloride, Figure 6, changed on standing. This change was accelerated by aeration of the solution. The curve, which originally dropped sharply above 300 mμ like the curve of rodiasine dimethiodide, shown in Figure 1, gradually developed a new peak at 342 mμ, similar to that of the ether-soluble alkaloid mixture, also shown in Figure 1. This new peak could not be removed by the addition of sulphur dioxide to the solution, and therefore was probably not due to the formation of a quinonoid derivative.

Treatment of dirosine with methyl iodide gave a mixture of products as might be expected from a secondary amine. Treatment with methyl iodide in the presence of alkali gave O, N-dimethyldirosine dimethiodide. Conversion to the methochloride gave a crystalline product which was hygroscopic and which gave somewhat inconsistent analytical results. The dimethiodide differs from that of O-methylrodiasine (and from the methiodides of the alkaloids represented by formula III) as shown below:

Alkaloid	Methiodide	
	$[\alpha]_D$	M. P.
O, N-Dimethyldirosine	+109°	266°
O-Methylrodiasine	+52°	304°
Formula III (±, ±)	±151°	182°
Formula III (±, ∓)	±0°	182°

The fully O- and N-methylated nonquaternary derivatives, O, N-dimethyldirosine and O-methylrodiasine, therefore must also differ from each other; and the original alkaloids, dirosine and rodiasine, must have different skeletal structures. O, N-Dimethyldirosine and O-methylrodiasine can be represented by the same formula only if they are diastereoisomers, and must otherwise be represented by different formulas. The physical properties of the methiodides rule out structure III both for O, N-dimethyldirosine and for O-methylrodiasine. The most likely structures for the latter compound have been shown to be IV, V, and VI.

Dirosine on treatment with diazomethane gave O-methyldirosine hydrochloride which gave the correct analyses for a completely O-methylated biscoclaurine alkaloid with one diphenyl ether linkage. The methylations with diazomethane were repeated a number of times, but the products obtained were not always the same. The results from seven methylations were as follows:

Run	Starting Material	Reaction Time	Yield	$[\alpha]_D$ of Hydrochloride	M. P. of Free Alkaloid
(1)	Dirosine	1 day	54%	+106°	—
(2)	Product (1)	1 day	70%	+107°	155°
(3)	Dirosine	10 days	10%	+107°	155°
(4)	Hydrochloride	1 day	54%	+103°	148°
(5)	Hydrochloride	2 days	40%	+102°	149° & 173°
(6)	Dirosine	2 days	67%	+105°	—
(7)	Product (6)	2 days	50%	—	176°

O-Methyldirosine (1) hydrochloride appeared to be incompletely methylated according to an equivalent-weight determination. Conversion to the free base and additional treatment with diazomethane gave O-methyldirosine (2) hydrochloride which gave the expected equivalent weight.

The hydrochloride (1) was obtained in needles, whereas the hydrochloride (2) was obtained in rhomboid platelets. Fractional crystallization indicated that the latter hydrochloride was a single product. However, the hydrochloride (1) gave the characteristic rhomboid crystals of the apparently completely methylated product and also needles of another material.

A considerable increase of the reaction time, in run (3), gave a considerable decrease in yield. The low yield might have been caused by the presence of methylene chloride or may have resulted from decomposition during a chromatography of the products. The hydrochloride (3) appeared to be identical with the hydrochloride (2).

The major product from the above chromatography was eluted later than the O-methyldirosine (3). The major product could thus have contained incompletely methylated material, but a new treatment with diazomethane gave no additional crystalline hydrochloride. There was also a possibility that the by-products which were not completely soluble in hydrochloric acid were formed by oxidation, for example, to amine oxide derivatives. However, catalytic hydrogenations, which normally would convert amine oxides to the original amines, gave no crystalline alkaloid hydrochlorides.

In the fourth methylation, dirosine hydrochloride was treated directly with ethereal diazomethane in order to prevent the losses which normally occurred in the conversion of the hydrochloride to the free alkaloid. The hydrochloride (4) could be obtained as the characteristic rhomboid platelets, but it also contained other material which was obtained in the form of needles. This product had a lower specific rotation and gave a free alkaloid having a lower melting point than the corresponding products obtained in runs (2) and (3).

The above methylation was repeated with double the reaction time to avoid possible incompleteness of the reaction. Run (5) did not give the characteristic hydrochloride platelets previously obtained; it gave a product which again had a low specific rotation, as did the hydrochloride (4). In addition to the lower-melting needles of free alkaloid, this product gave rosettes of a new higher-melting free alkaloid.

To prevent any influence on the reaction by employment of the hydrochloride as the starting material, free dirosine again was methylated. The resultant hydrochloride (6) did not give the rhomboid platelets. Conversion to the free base and a further treatment with diazomethane gave the hydrochloride (7) which also did not give the rhomboid platelets. O-Methyldirosine (7) was different from that produced in previous methylations of dirosine (m.m.p. 155°); instead, it was the same as the high-melting product obtained in the direct methylation of dirosine hydrochloride (m.m.p. 176°).

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Three different alkaloids were thus obtained in the methylation of dirosine. A consideration of the most likely formulas for dirosine, formulas IV, V, or VI, with one O-methyl group and one N-methyl group replaced by hydrogen, gives no reason why so many products should be formed. It is, however, difficult to picture any other structure which would be expected to yield such a variety of methylation products.

The possibility that a different product might be obtained in the methylation of dirosine and of the hydrochloride, and the possibility of the methylation of the secondary amino group were considered. Secondary amino groups do not normally react with diazomethane, however fluoroborate salts of secondary amines are known to react with diazomethane to give the tertiary amines. There is thus a possibility that the hydrochloride might react similarly.

The methylation of a tertiary amine by diazomethane to produce a quaternary derivative apparently has not been reported in the literature. The reaction of a tertiary amine hydrochloride with diazomethane could conceivably proceed in the following directions:

(1) $R_3N \cdot HCl + CH_2N_2 \rightarrow R_3N + CH_3Cl + N_2$

(2) $R_3NH^+Cl^- + CH_2N_2 \rightarrow R_3NCH_3^+Cl^- + N_2$
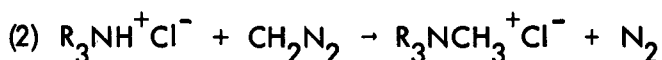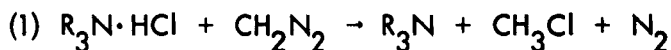
In the first of these reactions the diazomethane, in effect, converts the hydrochloride to the free base by reacting with the hydrochloric acid to form gaseous reaction products. In the second reaction a methochloride is formed, which would precipitate silver ions. In a trial experiment, the products of the reaction of diazomethane with dirosine hydrochloride gave no precipitate with silver nitrate solution, and therefore no methochloride had been formed.

It is possible that the product from run (2), which gave the expected analytical results, was O-methyldirosine hydrochloride, and that the lower melting product from runs (4) and (5) was O, N-dimethyldirosine in which the secondary amino group was also methylated. There is, however, no ready explanation for the formation of the alkaloid melting at 176°. It was considered that this material might be a different crystalline modification of the material melting at 155°, but recrystallizations of these products, in each case with seeding by the other alkaloid, gave no changes in melting points.

The free alkaloid methylation products from run (2) and from run (4) were treated with sodium in liquid ammonia to cleave the diphenyl ether linkage. The reaction products were extracted with ether and sodium hydroxide to give the

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"phenolic" cleavage products and a slightly larger quantity of "nonphenolic" products. Both cleavage products of methylated dirosine (4) gave positive phenol tests, as did the cleavage products of methylated dirosine (2). The infrared phenolic absorption peaks of the latter two "phenolic" and "nonphenolic" products were at 3550 cm^{-1} and at 3525 cm^{-1}, respectively, indicating the presence of phenolic groups without internal hydrogen bonding, [90] as might be expected of the cleavage products and as opposed to the 3360 cm^{-1} peak of dirosine.

Paper chromatography of the cleavage products obtained from methylated dirosine (2) gave long streaks indicating the presence of a number of compounds. No further studies of the cleavage products were made. The vapor-phase chromatography of papaverine was attempted with a Beckman CG-2 gas chromatograph, but this material could not be eluted from the column, and the possibility of separating coclaurine derivatives with the same instrument was thus very remote. The separation of the cleavage products might be achieved by vapor-phase chromatography with some of the newer instruments available, or with the recently introduced technique of thin-layer chromatography.

Grundon[46,97] reported the isolation of a third alkaloid from greenheart bark, which he called ocotine. It was obtained from one extraction of bark but not from a second extraction. The tentative formula $C_{35}H_{38}O_6N_2$, with four methoxyl groups and one N-methyl group, was assigned and a specific rotation of +32° was reported. This alkaloid was different from any of the purified alkaloids isolated in the present study. Ocotine, on methylation, was reported to give O, N-dimethylocotine dimethiodide with a specific rotation of +100°. This value is similar to the specific rotation of +109° of O, N-dimethyldirosine, and it is therefore possible that Grundon's ocotine is an isomer of dirosine or a mixture which contains dirosine.

I.   Other Chemical Considerations

A number of difficulties were encountered which impeded the progress of the studies of the greenheart alkaloids. Some of these difficulties have been mentioned in the above discussions. One of the chief problems was the instability of the alkaloids. This instability led to considerable losses in the purification of the alkaloids and it was probably responsible for some of the low yields obtained in the reactions of the alkaloids.

The alkaloids resembled each other very closely in many of their properties and for this reason it was difficult to devise good methods for identifying the alkaloids and good analytical methods for determining the composition of mixtures of alkaloids. For the same reasons it was also difficult to develop good methods for the purification of the alkaloids and for determining the actual purity of the alkaloids after they had been obtained in reasonably pure states.

For some time the investigations were also hampered by the difficulty of obtaining good microanalytical results. Low carbon values were obtained, and it is of interest that low carbon values for biscoclaurine alkaloids have been reported by other investigators.[54,55] More recent microanalyses of greenheart alkaloids by Dr. W. Zimmermann of the University of Melbourne generally have been very reproducible.

Another problem was the relatively small quantities of purified alkaloids which were available. Dirosine, the most abundant of the alkaloids, was present to the extent of only 11% of the ether-soluble alkaloids, and some other alkaloids were available in less than one-tenth of this quantity. Twenty-five grams of purified material might be considered a considerable quantity in some areas of the chemistry of natural products in which stable compounds which may be readily purified and analyzed might be obtained. A similar quantity of dirosine was rapidly consumed in methylation studies, in the preparation of other derivatives, and in further purifications, without giving very large quantities of these derivatives.

One of the reasons for the small quantities of individual alkaloids obtained was no doubt the fact that so many components were present in the ether-soluble alkaloid mixture. To reduce the number of components present, the mixture of alkaloids was O-methylated. Some related alkaloids with similar skeletal structures but differing in the number or position of free phenolic groups should thus have been converted to the same products. Gradient elution chromatographies of the O-methylated alkaloid mixture, followed by crystallization of the hydrochlorides of the fractions obtained, gave very little crystalline product. O-Methylation of the alkaloid mixture, followed by N-methylation, should further reduce the number of components present by eliminating possible differences at the amino portions of the alkaloids. However, the chromatography of the O,N-methylated alkaloids gave no separation and only a single peak instead of a few well-defined products.

It was expected that the residues of the chromatography fractions from which crystalline alkaloid hydrochlorides had been obtained should contain additional amounts of some of the alkaloids which had been characterized. Particularly, it was expected that some of the fractions from the second large-scale chromatography should have given additional amounts of alkaloids H, I, and J on further purification. Fraction 6 of this chromatography, which corresponded in cumulative yield to fraction 6 of the chromatography listed in Table I, was therefore chromatographed again with a gradient eluent. The fractions were obtained as the hydrochlorides, and their crystallization was attempted. Some crystals were obtained, but the yields were very small. It thus appeared that additional decomposition had taken place, and that the chromatography of crystallization residues from the other chromatography fractions would also be fruitless.

In the absence of other good methods of characterization of the alkaloids, such as, for example, an effective paper chromatographic method, the distribution coefficients proved very valuable in the identification of the alkaloids. The distribution coefficients of the alkaloids and of their derivatives varied over a range of 0.1 to 10 (for acetate buffer, pH 4.17-chloroform). The distribution coefficients were found to decrease by a factor of approximately 25 upon converting a phenolic compound to the corresponding nonphenolic compound.

Differences in the distribution coefficients of phenolic and nonphenolic alkaloids could be due to differences in the solubilities in water or to differences in the basicities. If the basicity were the predominant factor, a nonphenolic alkaloid, being more basic, would tend to go into the upper buffer layer and would, therefore, have a higher distribution coefficient. If the solubility were the predominant factor, the phenolic alkaloid, which can undergo hydrogen bonding and would therefore be more water-soluble, would have a greater tendency to go into the aqueous layer and would therefore have a higher distribution coefficient. Since the phenolic alkaloids have higher distribution coefficients than the corresponding nonphenolic alkaloids, the solubility appears to be more important than the basicity.

The above relationships and additional relationships are brought out in Table XI. A decrease in the distribution coefficient was also observed upon converting a secondary amino group to the corresponding N-methylated tertiary amino group. This change, by a factor of approximately 4, presumably again was due to a difference in solubility in water. The distribution coefficients of the alkaloids in Group A were smaller, again by a factor of approximately 4, than those of the corresponding alkaloids with equal numbers of phenolic and secondary amino groups in Group B.

As pointed out in Section IV. C, fractions 1 and 2 of the chromatography must contain alkaloids which are different from those already isolated. A consideration of the low distribution coefficients of these fractions, as shown in Table I, and a consideration of the representative distribution coefficient values, as shown in Table XI, indicates that the major alkaloids in fractions 1 and 2 must be nonphenolic alkaloids. A nonphenolic character would be consistent with their position at the beginning of the chromatogram since nonphenolic alkaloids are less polar than phenolic alkaloids.

It would be of interest to compare the alkaloids isolated from greenheart with other alkaloids isolated from other plants in the genus Ocotea. However, very little information on such alkaloids is available in the literature. The alkaloid ocoteine was isolated from Ocotea puberula by Iacobucci.[105] Although the structure of this alkaloid has not been unequivocally proven, it appears to be an aporphine alkaloid

related to the thalicmine (XXXVI).[106] (Ocotea rubra is another species of wood which is comparatively resistant to marine borer attack. However, this wood is reported to contain negligible quantities of alkaloids.[63]) No further studies of the alkaloids from other Ocotea species have been published.

Table XI. Distribution Coefficients

Functional Groups		Group A Alkaloids and Derivatives		Group B Alkaloids and Related Alkaloids	
NH	OH	Alkaloid	$\underline{K}$[a]	Alkaloid	$\underline{K}$[a]
0	0	O-Methylrodiasine	—	O-Methyloxyacanthine	0.23
				Otocamine	0.39
				O-Methylocoteamine	0.47
1	0	O-Methyldirosine	0.09	O-Methylisoocoteamine	0.33
				Ocodemarine	0.51
				Hydroepistephanine-A	1.03
0	1	Rodiasine	0.56	Oxyacanthine	2.4
		Isorodiasine	0.59	Berbamine	3.7
1	1	Dirosine	2.8	Ocoteamine	10.5
		Norrodiasine	2.3	Isoocoteamine	9.1
				Demerarine	11.5

a/ Distribution coefficient in 0.5 $\underline{M}$ acetate buffer, pH 4.17 – chloroform.

XXXVI
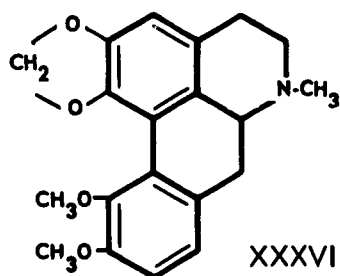
The genus Ocotea is part of the family Lauraceae. From other Lauraceae, aporphine alkaloids have been isolated. However, none of the alkaloids of greenheart show the characteristic properties of aporphine alkaloids. Two of these properties are comparatively high extinction coefficients at 283 mμ,[107] approximately twice as high as those of the greenheart alkaloids shown in Table IV, and a second absorption peak near 312 mμ which was not present in the purified greenheart alkaloids.

Besides the early references noted above, and the paper[46] and a previous preliminary announcement[97] by Grundon, there appears to be only one other report of recent work on the alkaloids of greenheart. This paper, by A. W. Sangster,[108] describes the separation of four greenheart alkaloids by electrophoresis on paper, but it gives no further information about the isolation or the chemistry of the alkaloids.

J. Resistance of Greenheart

Greenheart is probably the most widely used of the timbers which are naturally resistant to marine borer attack. However, there appears to be very little data available from which a potential user might determine the approximate service life of greenheart piling as compared to piling of other naturally resistant woods or compared to the service life of creosoted piling in the same location.

Long service life of greenheart piling has been reported for installations in many locations, but some premature failures have also been reported. It has been reported that greenheart was in good condition after 79 years of service in the gates of the Canada Dock in England.[109] Atwood and Johnson[5] presented a summary of service records for greenheart, collected prior to 1924, that includes greenheart which was reported to be in good condition after 61 years at Liverpool, England. However, the summary lists wide ranges in length of service and in conditions at the time of inspection. Failure in as little as five years is indicated. It was later pointed out by a number of authors that premature failures were often due to the selection of woods which actually were not greenheart.[4d, 6, 110, 111] In some other instances, premature failure of greenheart was shown to occur in fresh or brackish water.[4d, 5, 112, 113]

In the past, a number of different woods were sold as greenheart. Baldwin[6] showed that one installation which failed was not greenheart, as had been believed. He ascribed other failures to similar substitutions of woods which are not greenheart. A recent reference[110] warns against the acceptance of greenheart substitutes and it lists 15 species of wood which have been cited by Shu-Tien Li[111] as greenheart substitutes. One engineering handbook[114] lists a chemical test for the differentiation from greenheart of two other substitutes, greenheart from Dutch Guiana and yellowheart: The former gives a red color with potassium hydroxide and the latter gives a black color with ferric chloride, whereas Demarara greenheart gives neither of these reactions. It has been claimed that present rigorous inspection practically eliminates the possibility of obtaining substitutes when ordering greenheart from British Guiana.[4d]

The importance of salinity in affecting the resistance of greenheart has been stressed by a number of authors. Atwood and Johnson[5] had shown that one of the early failures which they had recorded was caused by a species of fresh-water **Teredo**

in the Ganges River. Record and Hess[4d] quoted a letter which mentioned the importance of high salinity and attributed failures in rhe Canal Zone to exposure in relatively fresh water. Edmondson[112] referred to mcre recent Australian exposure tests which indicated a relationship between salinity and resistance. To what extent the salinities were of importance and to what extent other ecological factors and the marine borer activities were of importance in the above observations is not known.

Work at the Naval Research Laboratory[113] has shown that for a number of woods, exposed in the Canal Zone, the resistance was much lower in Miraflores Lake than in the Pacific Ocean. The attacking species of marine borer, in the essentially fresh-water lake, was Teredo healdi. The woods which were less resistant in Miraflores Lake, and which included greenheart, had different mechanisms of resistance. It would not be expected that they would all be affected similarly by a reduction in salinity. The species and activity of the borers therefore appears to be more important than the salinity itself. These results thus do not support any implications that the inherent resistance of greenheart is affected by salinity.

Many comparisons of the resistance of greenheart and of various tropical woods have been made in the past, notably by Dutch and British authors. An extensive bibliography has been published, with 122 entries under this topic.[115] The results are difficult to compare because insufficient information is given about the size of the samples, the sample placement, the ecological conditions, and many other factors. Often only single samples were exposed.

More recent comparisons have been made by Wangaard[116] and by Edmondson.[2] Both authors have tested a considerable number of tropical woods. Although Baldwin[6] had stated that greenheart is the most resistant timber, both Wangaard and Edmondson tested a number of species which were more highly resistant to marine borer attack than greenheart. Edmondson had found that three samples of greenheart from different sources were quite different in their resistance to marine borer attack in Hawaii and that they were attacked by both Teredo and Limnoria. Wangaard does not specify which species of borer attacked his greenheart samples; however, it appears that most of the attack was caused by Bankia (of the family Teredinidae) and that there was little Limnoria damage even though Limnoria were active at the test site. (For those species of woods which were investigated by both Wangaard and Edmondson, the relative resistances were not always the same.) Some of the differences in the results are probably due to differences in the samples obtained from various sources and others may be due to differences in exposure conditions.

Greenheart panels exposed at Port Hueneme for five years showed moderate Teredo damage but no Limnoria damage. Similar panels exposed at Pearl Harbor were free from either Teredo or Limnoria attack but were removed from test after

about one year because of heavy <u>Martesia</u> attack. This failure might be considered premature because of the large size of the <u>Martesia</u> as compared to the 1/4-inch panel thickness. The testing of other tropical woods was very limited and was not necessarily intended as a direct comparison with greenheart.

Very little information appears to be available on the comparative resistance of greenheart and of creosoted timber. Experiences have been published which indicate the superiority of greenheart under some conditions.[109, 117] However, the literature appears to contain no reports of exposure tests which have made comparisons under known physical and ecological conditions, and which have reported the type of attack suffered.

The great desirability of greenheart for waterfront construction has been disputed in discussions with other investigators. For example, it was stated that greenheart was very heavily damaged by <u>Teredo</u> attack in five years at a site on the Atlantic Coast. However, on the basis of questioning, it appeared that damage to creosoted piling by <u>Limnoria</u> at the same site and over the same period of time was comparable to that suffered by the greenheart. Similarly, after the recent construction of a new greenheart pier in Miami, a number of persons interested in preservatives expressed the private opinions that the pier would not last very long and that creosoted piling would have been better.

On the other hand, the advantages of greenheart are, of course, emphasized by greenheart suppliers who often cite experiences that would show the superiority of greenheart over creosoted piling. There are thus many opinions concerning the relative resistance of greenheart and creosoted piling with practically no useful comparative data. If a decision is to be made on whether to install, at a given site, either creosoted piling or naturally resistant timber, as for example greenheart, comparative data for such piling material under similar exposure conditions would be very desirable.

Exposure tests at Port Hueneme harbor were conducted with 1/4-inch panels of greenheart and of creosoted pine. The greenheart panels received moderate <u>Teredo</u> attack in 60 months, whereas the creosoted panels suffered moderate to heavy <u>Limnoria</u> attack in similar exposure periods. This comparison does not show the superiority of either greenheart or creosoted southern yellow pine, since each was attacked by a different species of marine borer.

In this particular comparison it is difficult to extrapolate the results to the comparative performance of full-scale piling. Such extrapolations would be more meaningful in the comparison of two chemical treatments or of two naturally resistant woods. The creosote content of the small panels was roughly comparable to that

of the outer treated portion of a southern yellow pine pile and not to the total creosote content of approximately 20 pounds per cubic foot. The inner portion of a treated pile generally has low resistance, whereas the resistance of greenheart is more uniform. Although it can be argued that Teredo produces a more insidious type of attack since it eventually riddles the inside of the piling, it can also be pointed out that Teredo growth is quite stunted in greenheart. Furthermore, heavy Limnoria attack will ultimately destroy the creosoted outer portion of a treated piling and will give rise to rapid destruction by Teredo.

At Pearl Harbor the greenheart panels suffered no attack from Limnoria or Teredo, but, as was mentioned above, they were removed from test after about one year because of heavy Martesia attack. Creosoted southern yellow pine panels suffered no Martesia attack but half the panels failed in one and one-half to four years and all but one failed before five years because of heavy Limnoria attack. Again it is not possible to make extrapolations for full-sized piling, not only because of the reasons cited above, but also because of the effect of the Martesia on the small greenheart panels. It is expected that the comparative destruction by Martesia would be much less in full-sized piling.

The resistance of greenheart has widely been claimed to be due to the toxic alkaloids which it contains.[2, 4-7, 109, 112, 115-117] The resinous ingrowths and the texture and hardness of the wood have been claimed to be contributing factors.[6] It has been suggested that the high density of the wood is not a prime factor since there are denser woods which are less resistant;[116] and similarly, hardness is not a prime factor. The resistance of tropical woods to marine borer attack is often associated with a high silica content, but the silica content of greenheart is very low and, therefore, is not a contributing factor.[118, 119]

In the Laboratory's tests, the toxicity of the greenheart alkaloids to Teredo diegensis larvae was found to be of approximately the same order of magnitude as the toxicity of creosote. Furthermore, the ether-soluble alkaloids gave considerable protection to southern yellow pine when impregnated into this wood. The impregnated panels showed no trace of Teredo attack after ten months, whereas untreated panels had long failed by this time. It thus appears quite probable that the toxicity of the alkaloids is an important factor, and possibly the chief factor, in the resistance of greenheart to Teredo attack.

The toxicity of the alkaloids is not sufficient to prevent all molluscan attack. Martesia have caused considerable damage to NCEL test panels at Pearl Harbor. Teredo healdi have caused considerable damage to NRL test panels at Miraflores Lake. Whether the alkaloids are less toxic to these species, whether these species ingest correspondingly less wood, or whether they enter the wood in later stages of development, is not known.

The toxicity of the greenheart alkaloids to Limnoria was found to be much lower. Concentrations of 100 p.p.m. (0.01%) of the alkaloids in sea water did not kill the animals in four days. No direct comparisons can be made between the short-term exposures in sea water containing 0.01% of alkaloids and longer exposure to wood containing 0.4% of alkaloids. However, it does not appear from the above data that the toxicity of the alkaloids constitutes the most important factor in the resistance of greenheart to Limnoria attack.

The presence of alkaloids, on the other hand, appears to be an important contributing factor, as indicated by harbor test results with impregnated pine panels. Southern yellow pine panels, impregnated with 2% of the ether-soluble alkaloids from greenheart wood, were only lightly attacked by Limnoria in ten months, whereas untreated controls suffered heavy Limnoria attack and were removed from test after four months. The alkaloids thus have some deterrent action.

Toxicity tests with Teredo larvae showed that the toxicities of the alkaloids in Group A were slightly higher than those in Group B. However, the tests were not sufficiently quantitative or reproducible to establish the differences in toxicities between individual alkaloids within each group, and the effect of small structural changes on the toxicities could not be determined. The greenheart alkaloids were more toxic than several other alkaloids which were tested.

Because the alkaloids were not more toxic than creosote and because of their complicated structure, it did not appear worthwhile to synthesize related compounds as possible wood preservatives. The synthesis of biscoclaurine alkaloids has previously been accomplished [120] but it involves a complicated and long series of reactions. The synthesis of more simple related compounds would have been worthwhile if the toxicity of the alkaloids had been extremely high.

No definite information is available about the possible presence of other toxic components in greenheart. Greenheart sawdust was extracted with alcohol, ether, acetone, and water, and greenheart sawdust from which the alkaloids had previously been extracted with acid was further extracted with chloroform. The resultant extracts were not toxic to Teredo or Limnoria.

Small greenheart test panels extracted with chloroform, ether, or methanol had similar resistance to marine borer attack and suffered only slightly greater attack than the greenheart control panels. This similarity in resistance indicates that no toxic materials were extracted, but it does not indicate whether such toxic materials were not present, or whether, due to the impervious nature of the greenheart wood, such materials were present but were not extracted.

Greenheart panels extracted with sea water show a decrease in marine borer resistance. This decrease in resistance may have been due to chemical degradation of the wood to a softer and less resistant material by the action of the slightly alkaline boiling sea water. Panels extracted with boiling acetic acid showed increased resistance. These panels showed evidence of physical changes and it is possible that acetylation of the wood was partly responsible for the increased resistance. The increased resistance of acetylated wood has been reported by others. [121]

It was observed that greenheart alkaloids prevent the precipitation of silver and copper salts, probably by complex ion formation. The possibility existed that copper might be retained in greenheart by the alkaloids which were present. Spectrographic analysis of samples of greenheart sawdust and greenheart bark indicated that the maximum percentage of copper present was about one part per million. It is possible that this amount is significant, but no conclusive information is available.

Although the repellent action of the alkaloids does appear to contribute to the resistance of greenheart to Limnoria attack, other factors must also be important. The extent to which these various other factors, such as the presence of resinous material and the presence of other repellent chemical constituents, contribute to the resistance of greenheart to Limnoria attack is not known.

Even though greenheart is noted especially for its resistance to Limnoria attack, very little is yet known about the reason for this resistance. As noted above, Edmondson[2] has found that three samples of greenheart which he exposed were quite different in their resistance. If such samples of different resistance were available, a comparison of the differences between these samples might shed new light on the reasons for the resistance of greenheart to Limnoria attack. If such reasons were known, it should be possible to develop tests which could readily differentiate between highly resistant and less resistant samples. Such tests would be valuable in predicting the expected serviceability of new naturally resistant piling.

V. CONCLUSIONS

1. The ether-soluble alkaloids of greenheart, which had been believed to be highly toxic to marine borers and to be responsible for the resistance of greenheart to marine borer attack, were in fact found to be very toxic to <u>Teredo</u> larvae. The ether-insoluble alkaloids were found to be much less toxic.

2. The ether-soluble alkaloids impregnated into southern yellow pine prevented <u>Teredo</u> attack during a ten-month exposure period. The ether-soluble alkaloids are therefore likely to be the chief factor in the resistance of greenheart to <u>Teredo</u> attack.

3. Greenheart alkaloids were found to have a low toxicity to adult <u>Limnoria</u>, even though generally greenheart is noted especially for its resistance to <u>Limnoria</u> attack. Any important contributing factor of the alkaloids of greenheart, therefore, would not appear to be primarily due to their toxicity but would rather be due to other deterrent effects.

4. The ether-soluble alkaloids impregnated into southern yellow pine greatly reduced the amount of <u>Limnoria</u> attack. In spite of their low toxicity, these alkaloids thus exerted some protective action against <u>Limnoria</u> attack.

5. Although the deterrent action of the alkaloids does appear to contribute to the resistance of greenheart to <u>Limnoria</u> attack, other factors also must be important. The extent to which these various other factors, such as the hardness of the wood, the presence of resinous material, and the presence of other deterrent chemical constituents, contribute to the resistance of greenheart to <u>Limnoria</u> attack is not known. No readily extractible components toxic to <u>Limnoria</u> were found.

6. The toxicities of the greenheart bark alkaloids and of the greenheart wood alkaloids were essentially the same.

7. The ether-soluble alkaloids of greenheart bark were found to consist of a complex mixture of alkaloids and not, as had been believed, of the single predominant alkaloid "bebeerine."

8. The known alkaloid commonly called "bebeerine" is not present in greenheart. It appears that no purified alkaloids had been isolated previously from greenheart, and that rodiasine dimethiodide was the first pure alkaloid isolated from greenheart.

9. Eight purified alkaloids have been isolated from greenheart and names have been assigned. The alkaloids fall into two main structural groups. The alkaloids in Group A were slightly more toxic to Teredo larvae than those in Group B.

10. The alkaloids in Group A have properties which point to a bisbenzylisoquinoline structure with one diphenyl ether linkage. Included in this group of four alkaloids are: rodiasine, which has one phenolic group and four methoxyl groups; isorodiasine, which has a methoxyl group and a phenolic group interchanged with the corresponding groups of rodiasine; norrodiasine, which is a des-N-methyl derivative of rodiasine; and dirosine, which has a different skeleton but the same functional groups as norrodiasine.

11. Group B consists of four bisbenzylisoquinoline alkaloids with two diphenyl ether linkages, apparently all members of the oxyacanthine series. Included in this group are: ocoteamine (XV), a des-N-methyl-oxyacanthine; otocamine (XVII), the dimethyl ether of trilobamine; demerarine, apparently a des-N-methyl-repandine (XXVI or XXVII); and ocodemerine, apparently the corresponding methyl ether.

12. Because the structures of the alkaloids are quite complicated and because the toxicities of the alkaloids are not extremely high, it does not appear worthwhile to attempt the synthesis of closely related compounds as possible wood-impregnating materials. The possibility that more simple related compounds might be good wood-impregnating materials is, however, not excluded.

13. The resistances to marine borer attack of small panels of greenheart and of creosoted pine were comparable at Port Hueneme harbor. The former received moderate Teredo attack and the latter received moderate to heavy Limnoria attack in approximately five years. At Pearl Harbor the small greenheart panels failed prematurely because of heavy Martesia attack. No other tests designed to determine the relative resistance of greenheart as compared to the resistance of creosoted wood are described in the literature.

VI. ACKNOWLEDGMENTS

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Appendix A

A SYSTEM FOR PRODUCING GRADIENT ELUENTS

A gradient eluent changes slowly in composition without any abrupt changes in composition ever taking place. Some of the theoretical advantages of employing gradient eluents in chromatography have been discussed,[78-81] and a number of methods for producing gradient eluents have been described.[78, 79, 82, 83] However, none of the methods described were well suited for producing eluents in quantities of 25 liters or more, as required for the large-scale separations of the greenheart alkaloids, nor were they designed to give an eluent of the desired exponential composition curve.

Of the previously described methods, the one employing the device A, Figure 11, gave the curve which was most similar to that desired. The concentration curve A is obtained when the volume ratio of the containers in device A is 1:10, and curve C is the desired curve. The total capacity of device A must be as large as the total volume of eluent which is to be produced.
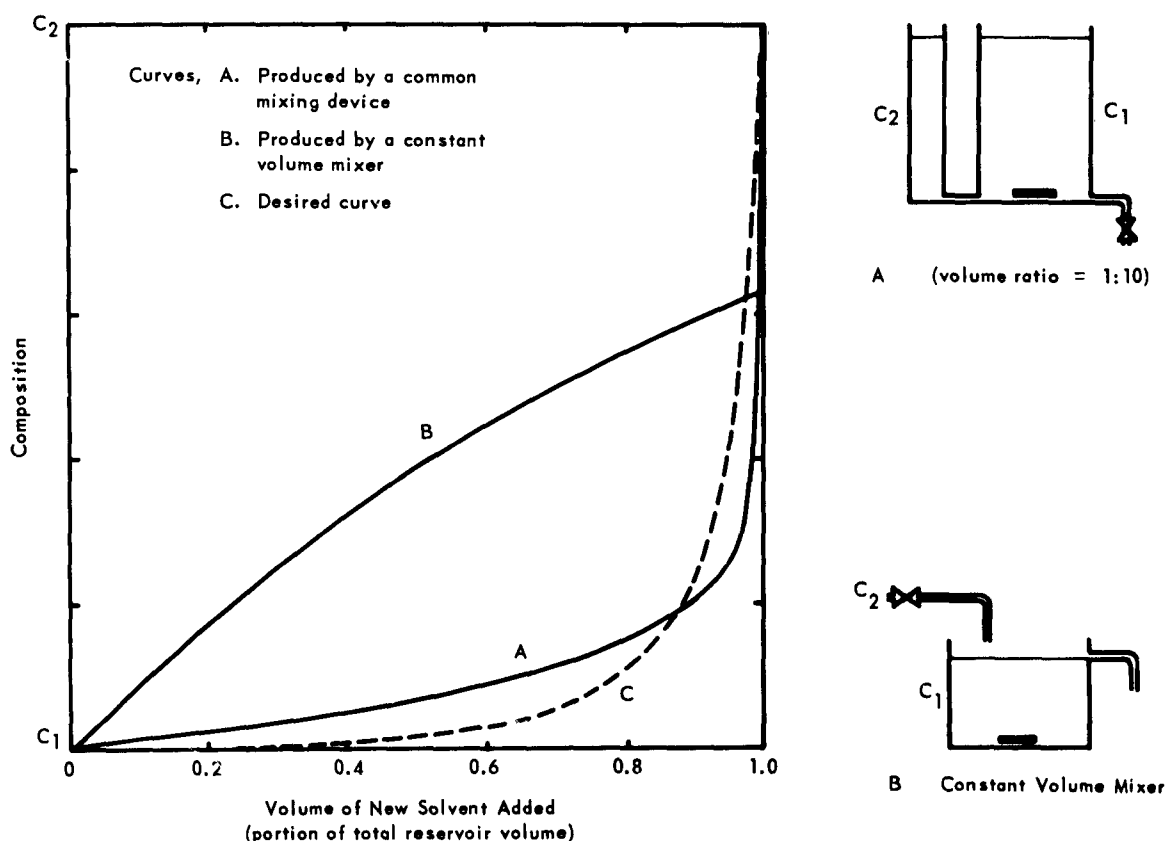
Figure 11. Devices for producing gradient eluents.

A versatile method for producing gradient eluents was devised, with the aid of a constant-volume mixer. The latter device had previously been employed to obtain gradient eluents with convex (rather than concave) composition curves and to obtain gradual transitions in eluent composition. Although the change in composition of the overflow of the constant-volume mixer follows a logarithmetic curve, as shown in curve B of Figure 11, the first portion of this curve approximates a straight line. By frequently changing the composition of small portions of new solvent added to the mixer, it is possible to produce an eluent having a composition curve which approximates, by a series of nearly straight lines, almost any desired shape.

The mixing system employed is shown in Figure 12. It consists of a three-necked flask with an inlet tube reaching near the bottom of the flask, with an efficient stirrer, and with an overflow or outlet going to the chromatography column. New solvents were added from a stoppered separatory funnel which maintained a constant liquid level in the mixing chamber. The 5- and 2-liter, three-necked flasks employed had capacities of 5.5 liters and 2.2 liters, respectively. For the production of small quantities of gradient eluents, a slightly different mixing system having a capacity of 100 ml. was constructed.

The composition curve of the overflow produced by the constant-volume mixer follows the equation: $y = 1 - e^{-x}$, where x is the partial volume of new solvent added, expressed as a portion of the mixer capacity (i.e., the volume of the new solvent added, divided by the mixer capacity), and y is the partial volume of new solvent present in the mixer and therefore the portion of new solvent present in the overflow. This equation is a simplified form of equations that have been given by others and is derived as follows:

If x and y are the partial volumes described above,

$$dy = dx - ydx$$

$$\frac{dy}{1 - y} = dx$$

$$-\ln(1 - y) = x + C$$

Since $y = 0$ when $x = 0$, $C = 0$. Thus,

$$1 - y = e^{-x}$$

$$y = 1 - e^{-x}$$
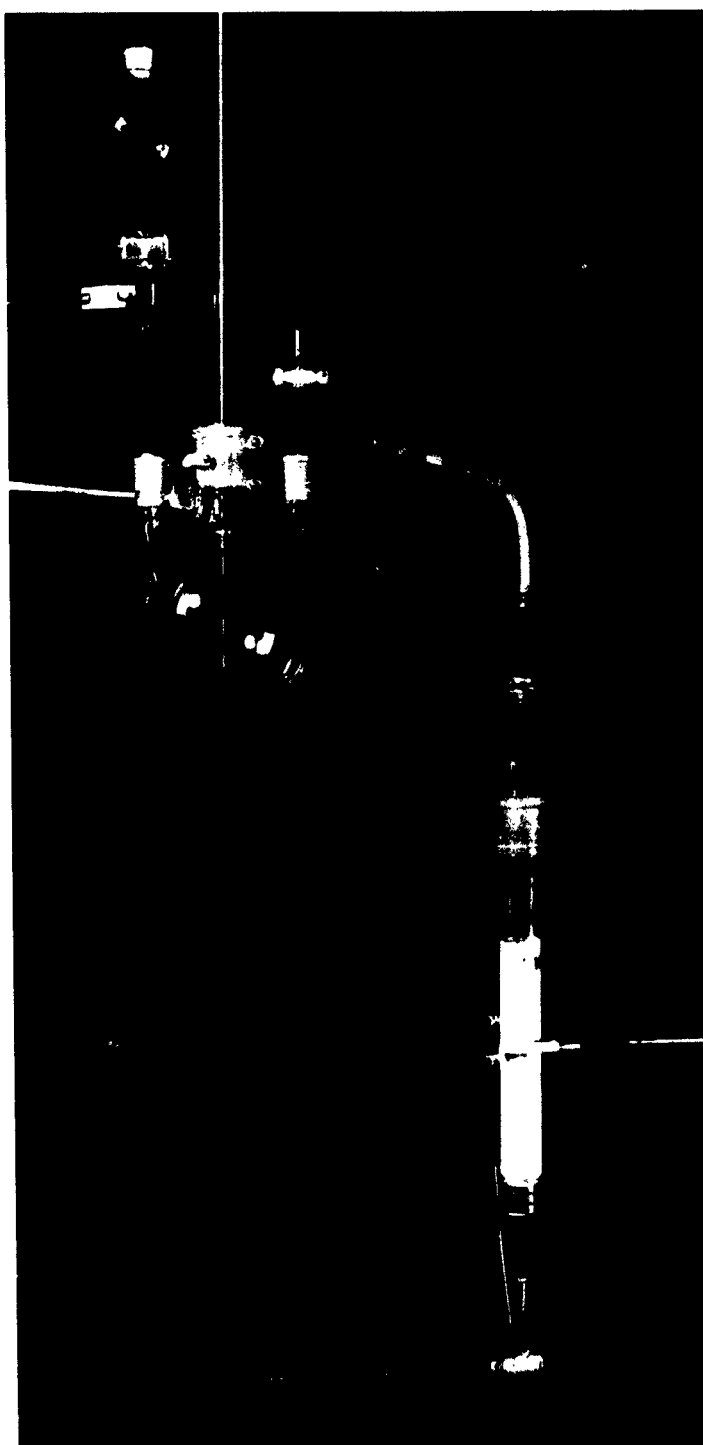
Figure 12. System for gradient elution chromatography.

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Table XII. Gradient Eluents Produced by the Mixing System

Cumulative Volume, liters	Composition of overflow[a] at end of addition of solvents[b] differing in concentration by factors of:							
	$\sqrt[2]{4}$		$\sqrt[3]{4}$		$\sqrt[4]{4}$		$\sqrt[5]{4}$	
2	(0.25%)	0.076	(0.25%)	0.076	(0.25%)	0.076	(0.25%)	0.076
4	(0.5%)	0.206		0.175		0.160		0.161
6	(1%)	0.448		0.314		0.264		0.245
8	(2%)	0.921	(1%)	0.523		0.399		0.345
10	(4%)	1.86		0.847	(1%)	0.582		0.471
12	(8%)	3.73		1.36		0.836	(1%)	0.632
14	(16%)	7.47	(4%)	2.16		1.19		0.850
16	(32%)	14.9		3.44		1.69		1.121
18	(64%)	29.9		5.46	(4%)	2.39		1.48
20			(16%)	8.68		3.39		1.95
22				13.8		4.80	(4%)	2.58
24				21.9		6.78		3.44
26			(64%)	34.7	(16%)	9.59		4.51
28						13.6		5.93
30						19.2		7.81
32						27.1	(16%)	10.3
34					(64%)	38.4		13.8
36								18.0
38								23.7
40								31.3
42							(64%)	41.0

a/ From a 5.5-liter mixing chamber.

b/ Consisting of 2-liter portions varying in concentrations, by the geometric factors designated, from 0.25% to 64% (of, for example, methanol in methylene chloride). (Some of the solvent concentrations are indicated in parentheses.)

When the volume of each new solvent portion added to the 5.5-liter mixer is 2.75 liters ($\underline{x}$ = 0.5), the calculated fraction of new solvent present in the mixer or in the overflow at the end of any addition is as follows: $\underline{y}$ = 1 − $\underline{e}^{-0.5}$, or $\underline{y}$ = 0.393. The remainder of the eluent, 0.607 of the total, is the solvent mixture present before the addition of the new solvent. Thus the eluent consisting of 2.75 liters of methylene chloride followed by 22 liters of gradient eluent, produced by adding 2.75-liter portions of 0.25%, 0.5%, 1%, 2%, 4%, 8%, and 20% methanol in methylene chloride, and then 100% methanol to the mixing chamber originally containing methylene chloride, follows the composition curve shown in Figure 5.

In other chromatographies involving large volumes of eluent, it was found more desirable to employ 2-liter portions of solvent and to vary the total amount of solvent employed, as well as the slope of the concentration curve, by selecting different ratios for the changes in concentration of successive new solvent portions. Employing ratios of the square root, cube root, fourth root, and fifth root of 4, and starting with 0.25% methanol and finishing with 64% methanol, gives cumulative volumes of 18, 26, 34, and 42 liters, respectively. Here $\underline{x}$ = 0.695 and $\underline{y}$ = 0.305. The calculated concentration of the methanol in the overflow at the end of each addition of new solvent is listed in Table XII. The curves of the eluents produced are shown in Figure 13. The eluent produced by adding 1-liter portions of 0.25%, 0.5%, ..., 32%, and 64% methanol in methylene chloride, and then methanol, follows the upper curve shown in Figure 13.
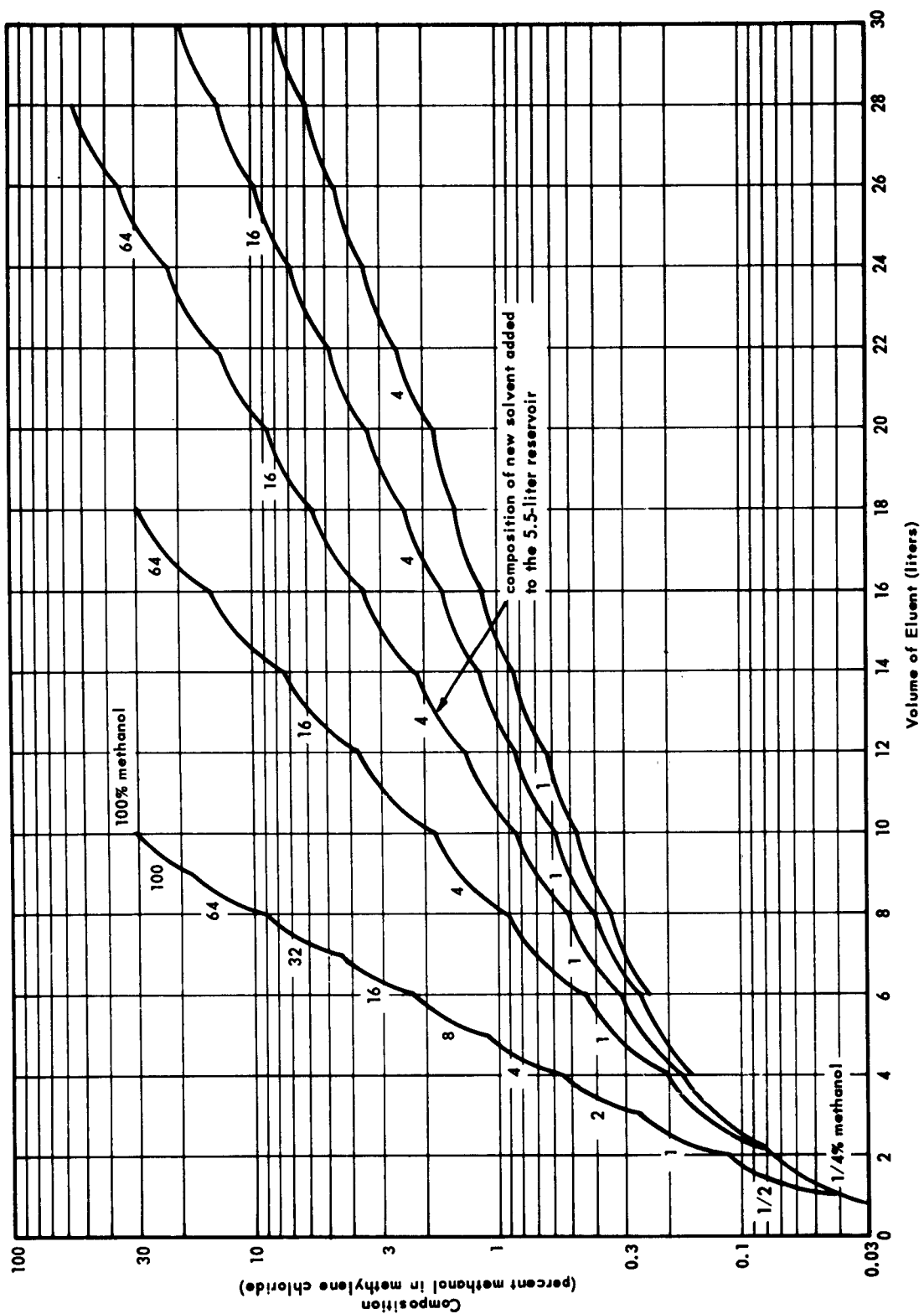
Figure 13. Eluents produced by the mixing system.

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Appendix B

INFRARED SPECTRA

Reproduced herein (Figures 14-20) are the infrared spectra of some of the alkaloid hydrochlorides discussed in the foregoing sections. These spectra were obtained with a Beckman IR-7 spectrophotometer and with samples in potassium bromide discs as described in Section III. J. 5a. Many other spectra were obtained but the differences in the spectra were such as not to be readily apparent in small reproductions, and they are therefore not included. Many comparisons were made with spectra of chloroform solutions and with spectra covering only a portion of the wavelength scale. These again are not reproduced here.

139
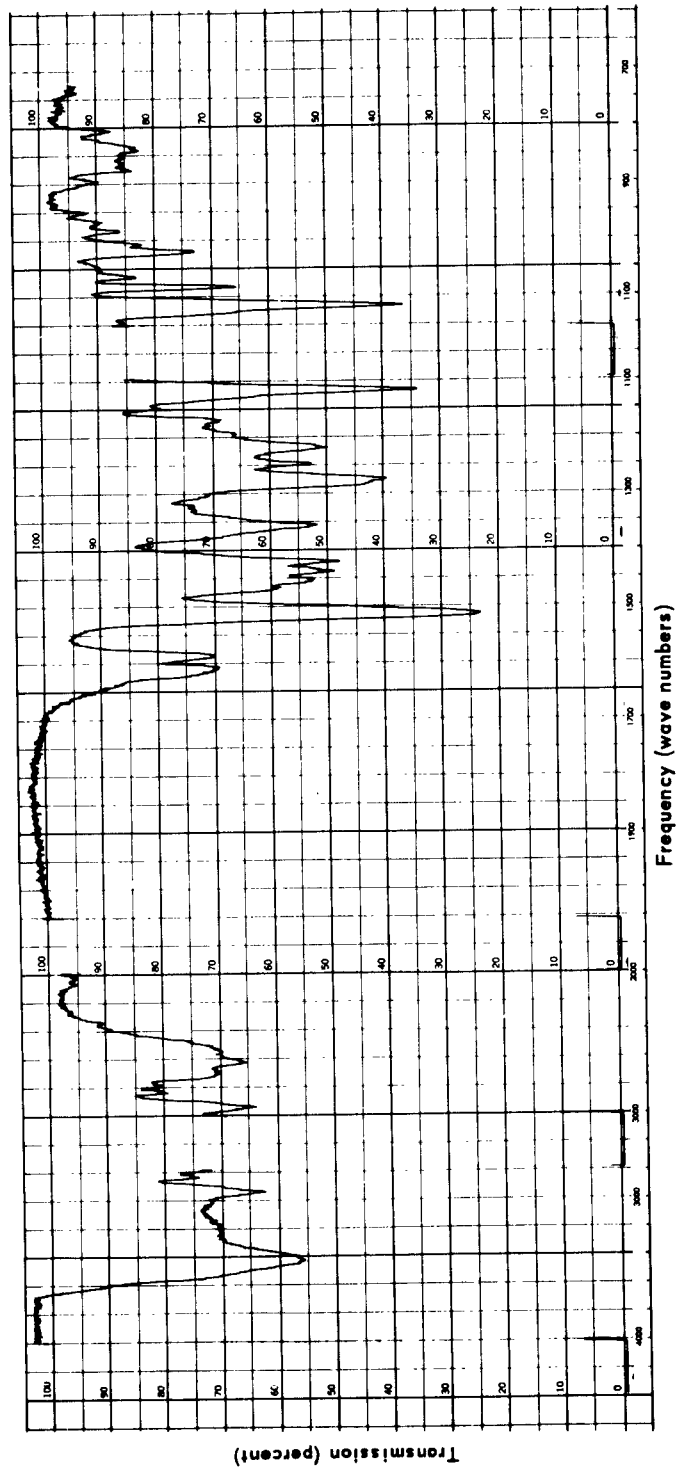
Figure 14. Infrared spectrum of oxyacanthine hydrochloride.

Frequency (wave numbers)

Transmission (percent)

140
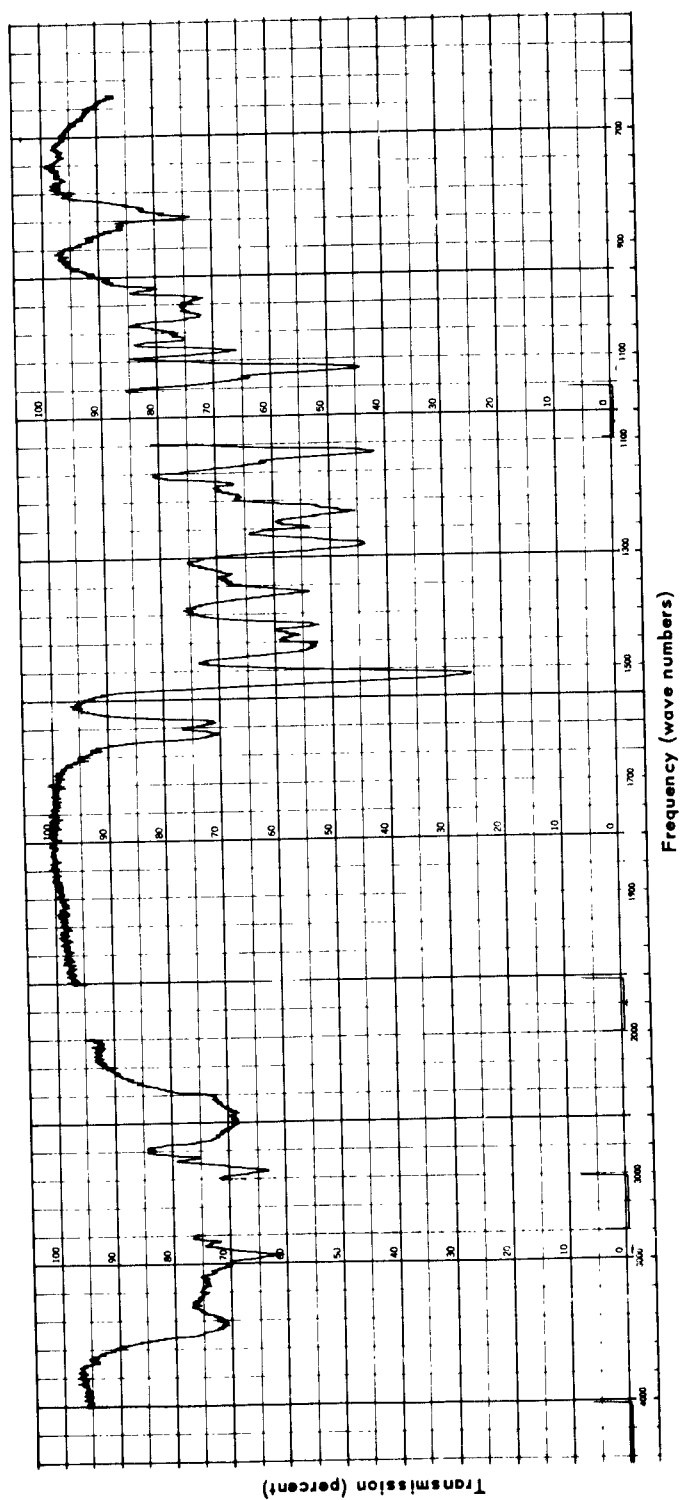
Figure 15. Infrared spectrum of berbamine hydrochloride.

141
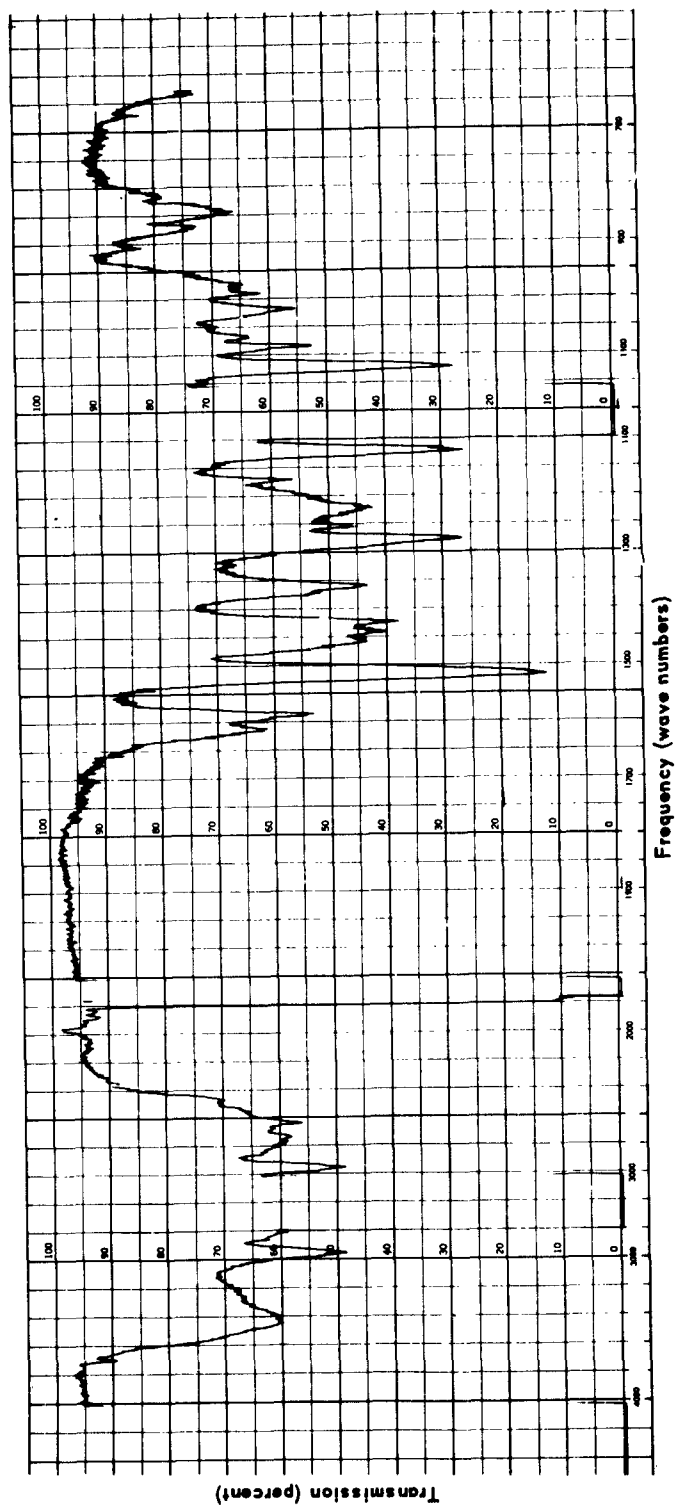
Figure 16. Infrared spectrum of ocoteamine hydrochloride.

142
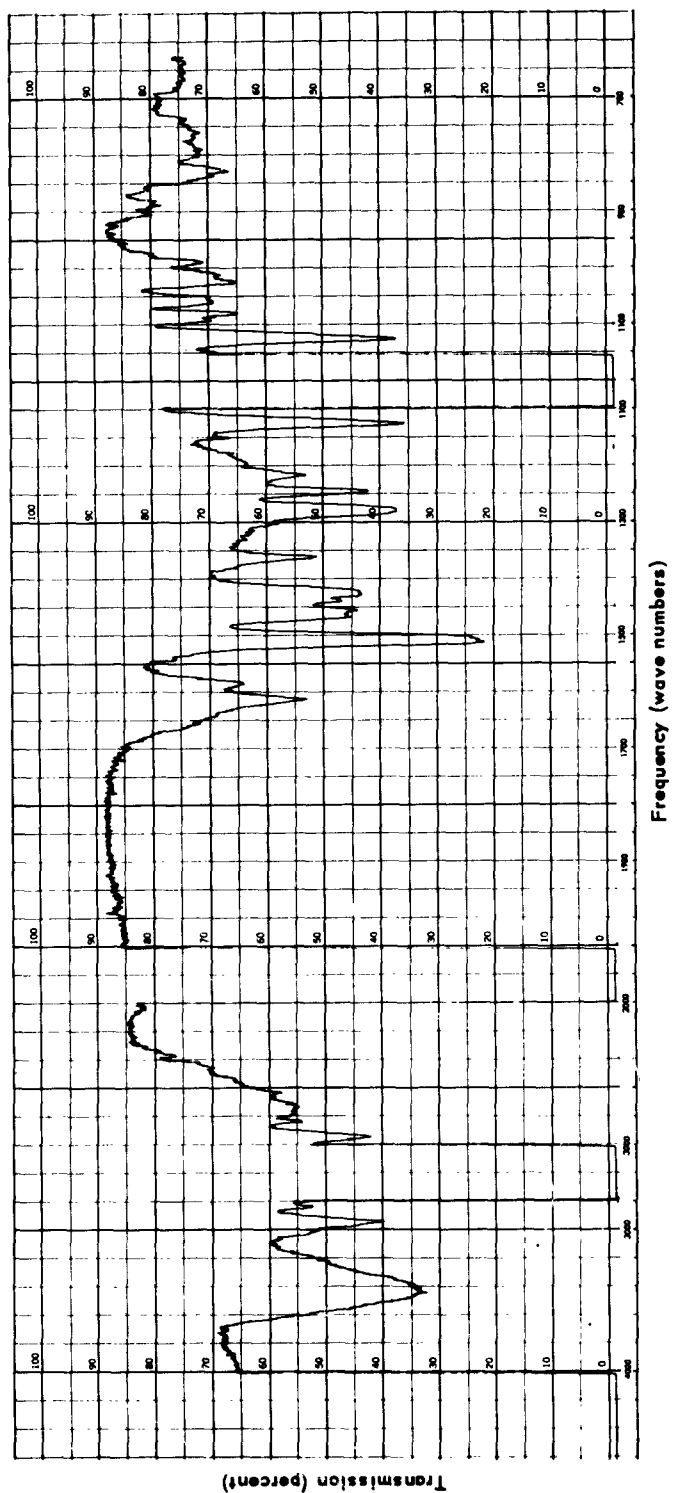
Figure 17. Infrared spectrum of dirasine hydrochloride.

143
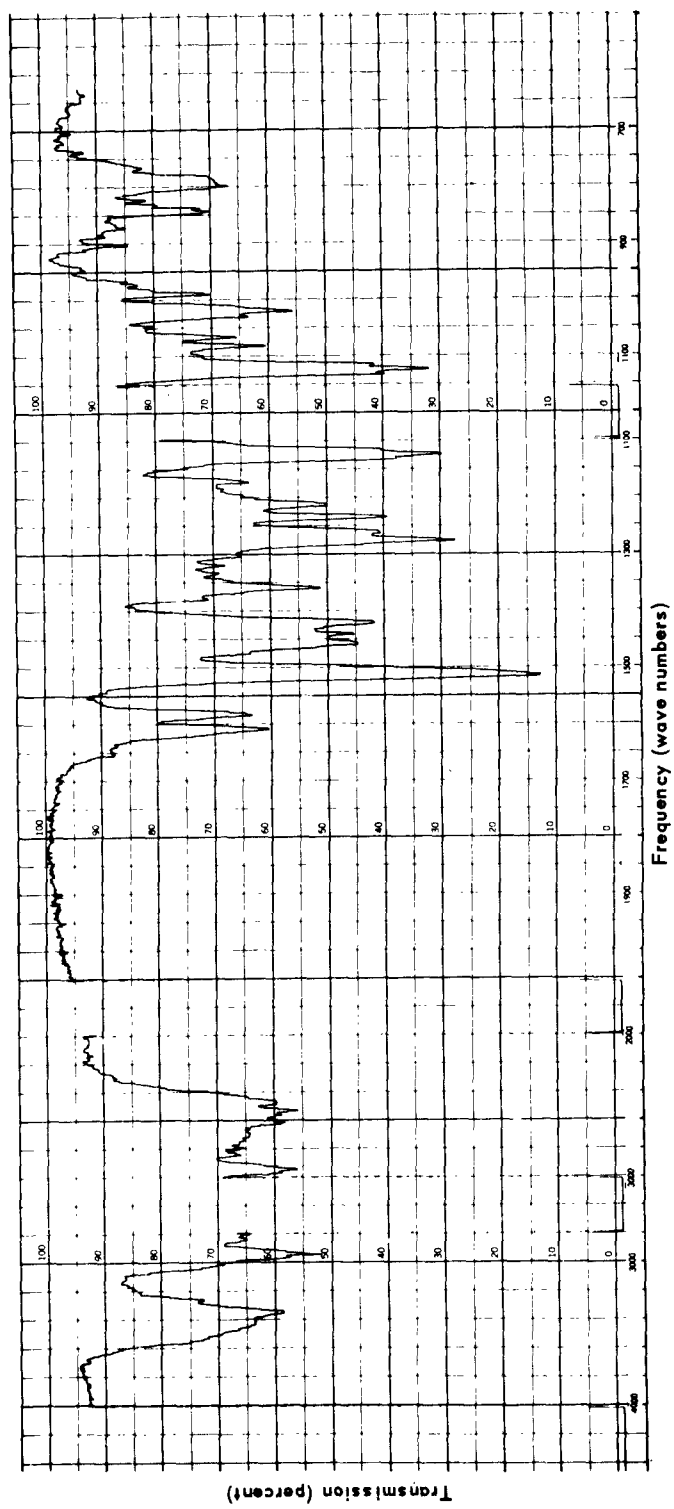
Figure 18. Infrared spectrum of otocamine hydrochloride.
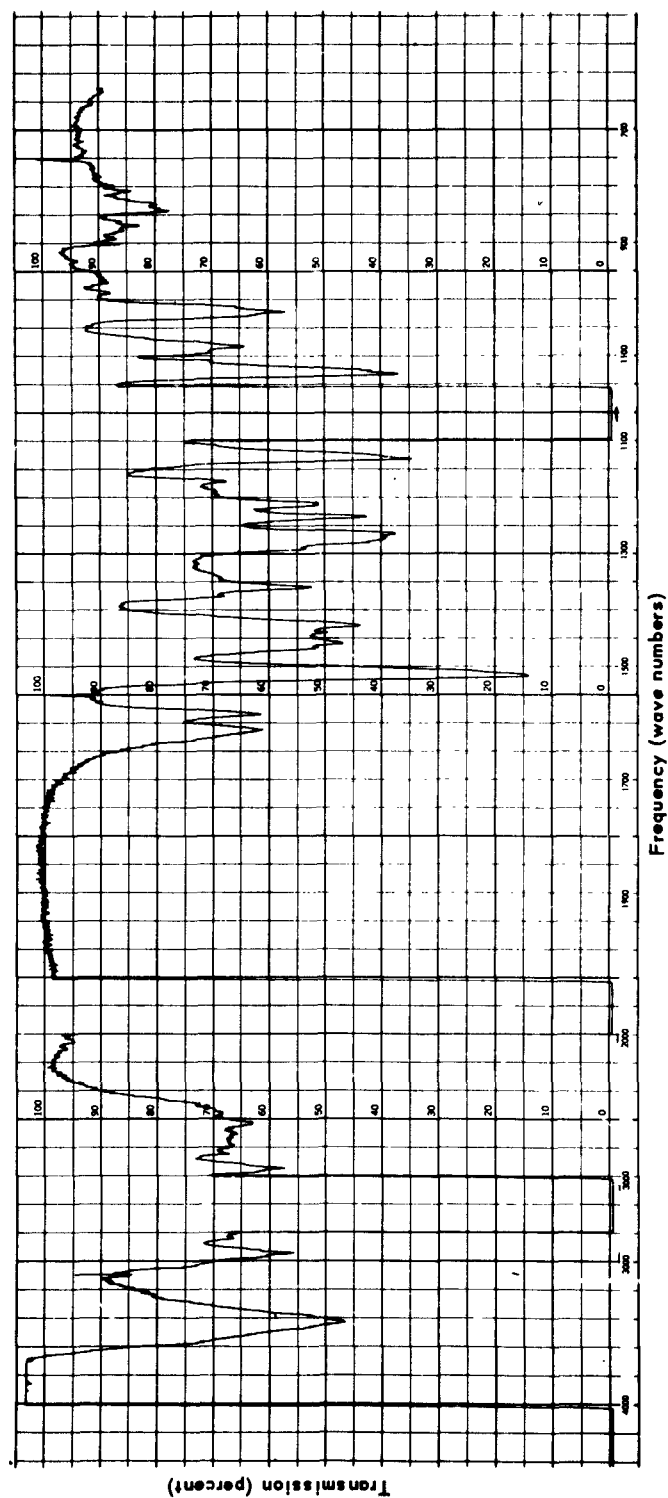
Figure 19.   Infrared spectrum of hydroepistephanine–A hydrochloride.

145
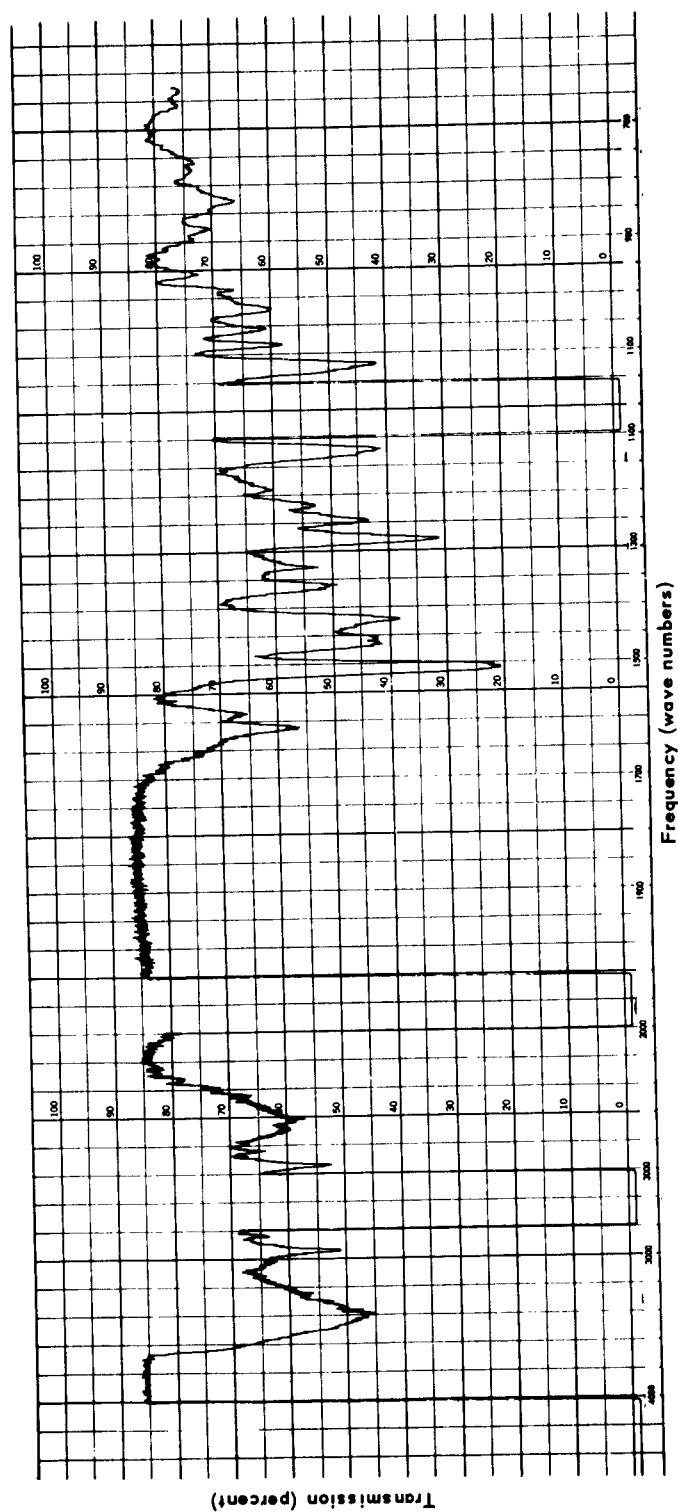
Figure 20. Infrared spectrum of rodiasine hydrochloride.

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U. S. Naval Civil Engineering Laboratory
Technical Report R-224
GREENHEART ALKALOIDS, by Peter J. Hearst,
Ph. D.
149 p. illus.   25 Mar 63   UNCLASSIFIED

Eight alkaloids have been isolated from
greenheart. Four of these (ocoteamine, otocamine,
demararine, and ocodemarine) are bisbenzyliso-
quinoline alkaloids of the oxyacanthine series.
The other alkaloids (rodiasine, isorodiasine,
norrodiasine, and dirosine) appear to be bis-
benzylisoquinoline alkaloids with one diphenyl
ether linkage. The above alkaloids apparently
are the first pure alkaloids isolated from green-
heart. The alkaloid "bebeerine," which was
believed to be the chief marine borer deterrent
in greenheart, could not be isolated. All the
isolated alkaloids are quite toxic to Teredo but
less toxic to Limnoria.

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